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DOCTOR OF PHILOSOPHY

Chemical synthesis of Leishmania lipophosphoglycan structures conjugated to a biotin moiety

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**Chemical synthesis of *Leishmania*
lipophosphoglycan structures conjugated to
a biotin moiety**

A Thesis presented for the Degree of Doctor of Philosophy

By

Christopher W. Edgar



December 2011

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Declaration

I hereby declare that the research described herein has been carried out and that this thesis is of my own work. This thesis has not been accepted in fulfillment of the requirements of any other degree or qualification. The research described herein was carried out in the College of Life Sciences, at the University of Dundee, under the supervision of Dr. A. V. Nikolaev.

Christopher W. Edgar

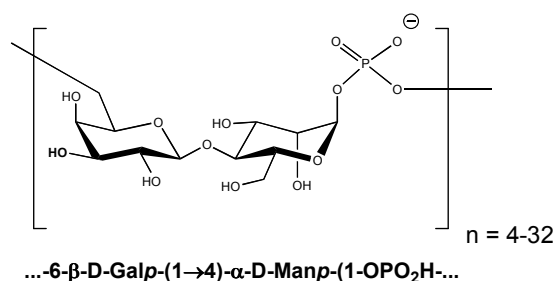
Certification

I hereby certify that Christopher W. Edgar, BSc Hons, has undertaken full-time research at the College of Life Sciences, University of Dundee, under my direction since October 2007, and that he has fulfilled the conditions of ordinance 14, so that he is qualified to submit this thesis for the degree of Doctor of Philosophy.

Dr. Andrei V. Nikolaev

Synopsis

Leishmaniasis, the disease caused by a genus of *Leishmania* protozoan parasite, is the second largest parasitic killer in the world (after malaria) responsible for an estimated 500,000 cases and 60,000 deaths each year worldwide. Visceral leishmaniasis infections (i.e., affecting internal organs like liver and spleen) are more complicated to diagnose due to the lack of visible symptoms. Current tests of the disease look for antibodies against the parasite, but these antibodies are expressed long after the infection. It has been found the *Leishmania* parasite excretes a unique phosphoglycan repeat unit:

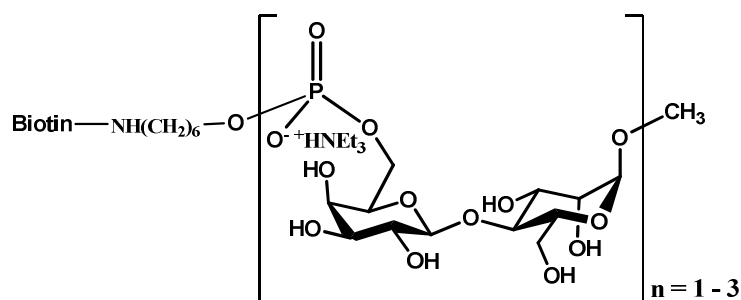


By looking for this repeat unit, the diagnostic test will be checking for an active infection in the patient. It has been found that there are monoclonal antibodies to detect this uniquely excreted phosphoglycan. This provides a basis for a test kit, the biomarker (i.e., the phosphoglycan repeats), and the way to detect the biomarker. For the construction of a dipstick style test or an ELISA format assay, a chemically synthesised repeat unit phosphoglycan is needed to act as a positive control in a dipstick test or as a known comparison in an ELISA.

The synthetic phosphoglycan structure must also be anchored to a solid surface for testing. Biotin will be used as the anchor. It has shown many different applications in biotechnology and its very high affinity to the avidin proteins make it very useful. A

6-aminohexanol spacer arm (i.e., a linker) will also be included in the synthetic structures.

After original attempts to introduce the biotin moiety to the repeat unit at the D-mannose 1-phosphate, it was decided to change the strategy and integrate the biotin moiety via linker and phosphate at the D-galactose unit. This meant performing chain elongation from the reducing end, with the first disaccharide unit being capped at the D-mannose anomeric position with a methyl group. Below are examples of the targeted biotinylated phosphoglycan structures, which have been prepared in this project.



All three prepared biotinylated phosphoglycan structures were tested in ELISA assay using monoclonal IgG antibodies that recognises the repeat unit structure. They showed different activities in terms of antibody recognition and binding. The largest compound containing three repeats demonstrated the strongest signal. This positive result provides a foundation for novel assay formations and further immunological work.

1. INTRODUCTION

1.1 Biological Introduction

1.1.1 Neglected Parasitic Diseases

Diseases such as malaria, leishmaniasis, African sleeping sickness (or African trypanosomiasis) and Chagas disease (or American trypanosomiasis) are all caused by insect-transmitted parasites. These diseases infect millions of people around the world and millions more are at risk of infection. Figure 1 gives an overview of the diseases their causative agents and the number of cases reported.

Protozoan causative agent	Disease	Number of cases
<i>Leishmania donovani</i> <i>Leishmania major</i> <i>Leishmania mexicana</i>	Leishmaniasis	12 million
<i>Trypanosoma brucei</i> <i>Trypanosoma brucei gambiense</i> <i>Trypanosoma rhodesiense</i>	African trypanosomiasis	300,000-500,000
<i>Trypanosoma cruzi</i>	Chagas' disease	16-18 million
<i>Plasmodium falciparum</i> <i>Plasmodium malariae</i> <i>Plasmodium ovale</i> <i>Plasmodium vivax</i>	Malaria	300 million

Figure 1 – Parasitic protozoan diseases [1]

1.1.2 Leishmaniasis

The origins of Leishmaniasis are unknown ^[2]. There are records of lesions similar to the ones caused by leishmaniasis on tablets from the library of Ashurbanipal, King of Syria, from the 7th century BC. These are thought to be derived from earlier texts from 1500 to 2500 BC ^[3]. It was first described in the modern era by Peter Borovsky, a Russian military surgeon who was conducting research into the etiology of an oriental sore in Tashkent, Uzbekistan. In 1898, he published the first accurate description of the causative agent, the parasite's relation to the host tissues and even referred to it as a protozoa. However, his results were published in a Russian journal with low circulation so were not internationally acknowledged during his lifetime ^[4]. In 1901 William Boog Leishman and Charles Donovan identified certain organisms in smears taken from the spleen of a patient who had died from "dum-dum fever" and called them trypanosomatids, which came to be known as Leishman-Donovan bodies ^{[5][6]}.

1.1.2.1 Leishmaniasis Epidemiology

Leishmaniasis is a poverty related disease affecting the poorest people who are suffering from malnutrition, are being displaced and have a weakened immune system. Leishmaniasis is also related to environmental changes such as

deforestation, dam building and new irrigation schemes causing migration of non-immune people into endemic areas^{[7][8]}. The disease is prevalent and endemic in the tropics and sub-tropics covering large areas of Bangladesh, Pakistan, India, Africa, the Middle East, South and Central America and along the Mediterranean.

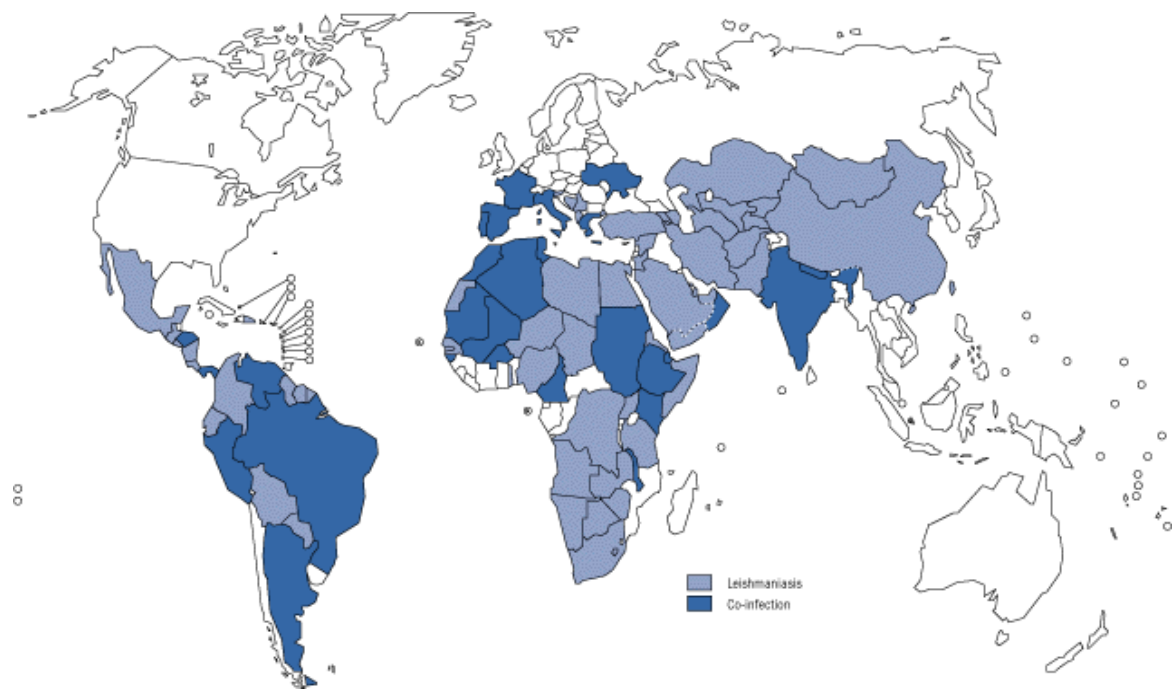


Figure 2- Geographical distribution of leishmaniasis (light blue) and co-infection with HIV (dark blue)

[1]

1.1.2.2 Disease Burden

Recently the public health impact of leishmaniasis has been re-assessed and it was found that previous estimates were very low compared to the actual burden of the disease. It is now estimated that there are 2 million cases annually with 1.5 million of the cutaneous form and 500,000 of the visceral form and with around 12 million people infected throughout the 88 countries currently affected ^[9]. More surprising is that only 32 of the 88 countries that are affected by the disease actually have to report leishmaniasis infection. The actual numbers could be dramatically different ^[7].

1.1.3 Pathology

Leishmaniasis can present in 3 main forms each dependent on the area of the body infected (Fig 3).



Figure 3- Leishmaniasis infections

1. Visceral leishmaniasis (VL), ^[10] ^[11] infects the internal organs of the patient attacking the liver and spleen. If left untreated VL is fatal.
2. Mucocutaneous leishmaniasis (MCL), ^[12] ^[13] infects the mucous tissue of the nose and mouth. MCL infections self-heal but result in massive tissue damage and permanent disfiguration.
3. Cutaneous leishmaniasis (CL), ^[14] ^[15] infects the skin of the patient and results in self-healing ulcers that permanently disfigure the skin.

Worldwide visceral leishmaniasis makes up for about 25% of infected patients. Of these cases 90% are found in India, Sudan and Bangladesh ^[16]. Temperature could play a part in the localisation of the parasite species in the host. Some species of the parasite causing cutaneous leishmaniasis cannot grow at the core body temperature while species causing visceral leishmaniasis can ^[17]. The parasitic species causing the *Leishmania* infections can be put into three large species groups and three less diverse groups ^[18]. The parasites in these groups all have a similar physical appearance but with differing clinical manifestations.

3 Large Groups:

1. *L. braziliensis* - cutaneous and mucocutaneous leishmaniasis in the Americas
2. *L. mexicana* - cutaneous leishmaniasis in the Americas
3. *L. donovani* – visceral leishmaniasis and 2 related members *L. infantum* in the Mediterranean and *L. chagasi* in the Americas.

The three small groups are *L. major* and *L. tropica* both causing cutaneous infections in Africa and the Middle East. *L. aethiopica* causes cutaneous infections in the North East of Africa.

1.1.4 Parasite life cycle

The parasite life cycle is a complex one surviving in the vector and the mammalian host and the many different environments this provides. The vector for the leishmania parasite is a certain species of sand-fly (Fig 4) from the genus *Phlebotomus*^[19].



Figure 4 – Sand-Fly vector for *Leishmania*

Although there are around 500 different species of sand-fly (phlebotomine) only around 30 have been found to be vectors of the leishmania parasites^[20].

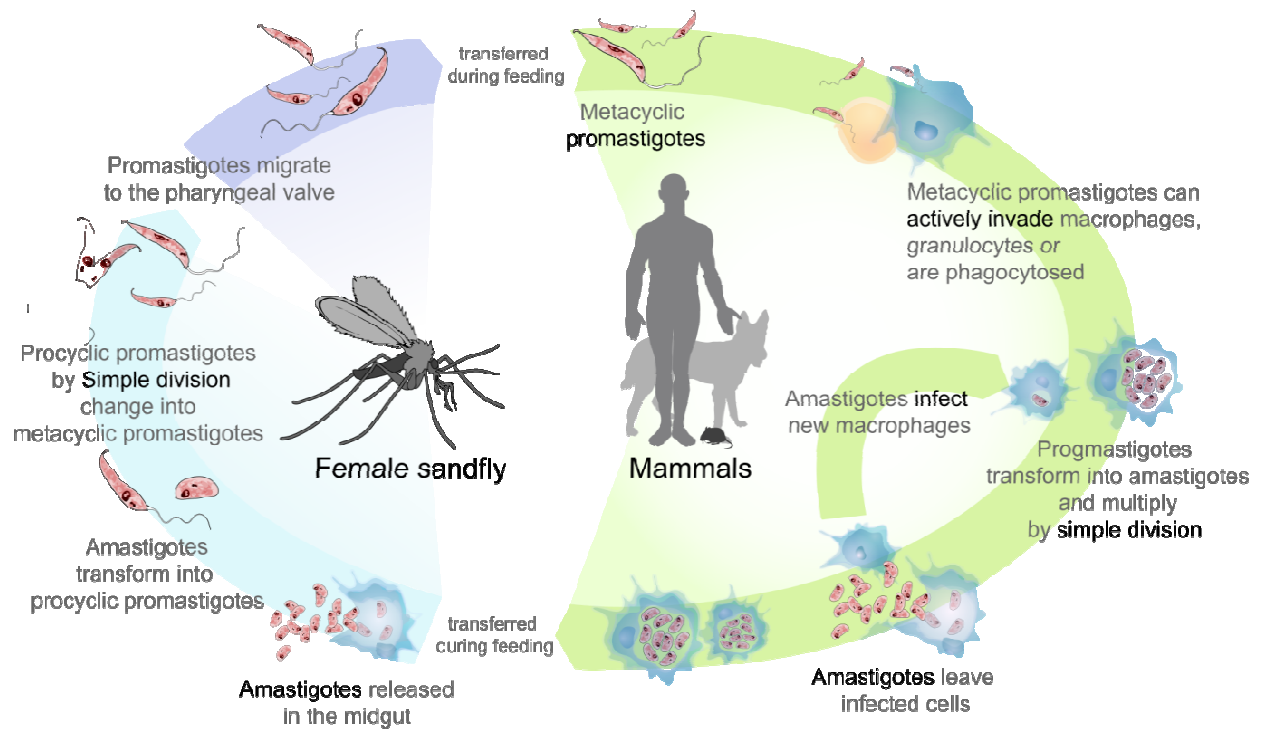


Figure 5 – The infectious cycle of *Leishmania*^[21]

Starting with a bite of an infected sand-fly into a non-infected host, the parasite promastigote metacyclic form is transmitted to the host. The parasite enters from vector to host with the anti-clotting factors which are injected normally by the sand-fly to ensure a steady blood flow. From here, the injected metacyclic parasites invade the host's macrophages. In these macrophages the parasite differentiates

into the amastigote form and multiplies. The cells break down leaving the parasites free to infect other cells. This process repeats until treatment clears the infection or death. When another sand-fly, this time uninfected, takes a blood meal from the infected host it will ingest some of the parasites released from the macrophages. These enter the fly and attach to the mid-gut of the vector. From here they transform back to the procyclic form, multiply and travel to the mouth of the sand-fly where they are ready to be excreted in the next blood meal. During its complex life cycle the trypanosomatidae exhibits 2 distinct morphological states^[22]. Promastigotes replicate in the midgut of the sand-fly vector and then transform into their infective metacyclic form. They are ingested into the macrophages, where they differentiate and proliferate as amastigotes in the phagolysosome compartment. Good reviews have been published on the *Leishmania*-sand fly interaction^[23], the transmission mechanisms of the metacyclic promastigote^[24] and the metabolism of amastigotes inside human macrophages^{[25][26]}.

1.1.5 Treatment

The most common treatment for leishmaniasis is a chemotherapy approach using pentavalent antimonials administered parenterally. The main compounds used are meglumine antimoniate and sodium stibogluconate, but the actions of these drugs are not totally understood^{[27][28]}. If the antimonials have no effect, alternative approaches are available using amphotericin B, pentamidine and paromomycin.

These approaches are expensive, moderately effective, toxic and the parasite is starting to show resistance^{[29][30]}. There is no vaccine for leishmaniasis but there are a number currently going through clinical trials^{[31][32][33][34]}. There is still a great need for treatments for leishmaniasis.

1.1.6 Diagnostics

Diagnosis of an active leishmaniasis infection can be tricky. In the cases of cutaneous and mucocutaneous infections this is straight forward as they present with skin lesions. Treatment can be immediate and the confidence in diagnosis is high. In the case of visceral leishmaniasis this is very different. Visceral leishmaniasis attacks the internal organs and if not treated in a timely manner is fatal. To complicate things further the symptoms caused by the infection are very similar to other diseases such as malaria^[35]. The longer the patient spends untreated the more at risk the community becomes as the patient will act as a reservoir for the disease. To make matters worse the medical staff want to be sure of the diagnosis before the start of treatment due to the toxic and expensive drugs they will have to administer for the treatment length of 3 weeks^[35].

1.1.6.1 Gold standard Test

The gold standard test (the best test) uses peripheral blood or aspirates from marrow, spleen, lymph nodes. These are gathered by biopsies of the specific area. The cells are then placed on a slide, stained and viewed under a microscope (Fig 6)^[37].

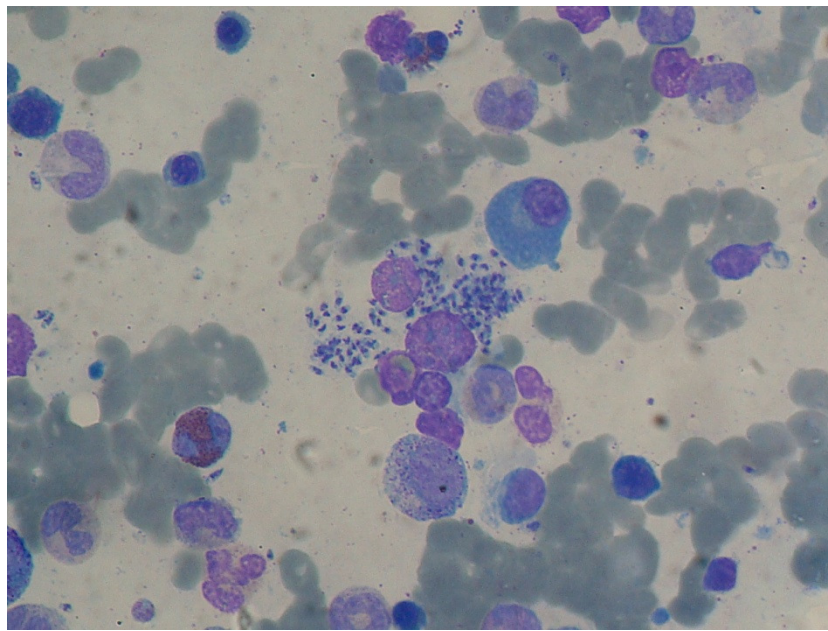


Figure 6 – Bone marrow aspirate smear of visceral leishmaniasis

These biopsy and microscope techniques are very effective but require sterilised conditions, high level of training, high tech equipment and after care. This would be no problem in the developed world, but as most visceral leishmaniasis cases are found in the developing world (India, Sudan and Bangladesh) this equipment and expertise are not available to the vast populations.

What is required is a robust simple field test kit that can be run anywhere and with results that can be trusted.

1.1.6.2 Latex agglutination test - KAtex

The test has been developed to provide accurate results for the infection of visceral leishmaniasis patients in the field. The general procedure for the test is below :

1. Collect patient's urine.
2. Take 1ml of urine and boil for 5 mins and allow to cool.
3. Add 50ul of the boiled urine to the reaction zone on the test plate.
4. Add 1 drop of the testing latex on to the sample of urine.
5. Stir both liquids to form homogenous mixture.
6. Tilt the plate with a rotating action – clockwise and anti-clockwise for 2 mins.
7. After 2 mins, read the degree of agglutination obtained.

The test works by using antibodies bound to the latex, these antibodies then bind to the antigen in the urine, with agglutination as a positive result. The results from the test can be read fairly quickly and can be done by eye, so no complicated machines are required. This test has drawbacks when it comes to the “field setting”. The boiling of the urine is possible in most settings but can cause problems in some. The tests themselves have to be kept at 4 °C and the kit itself is only for a minimum of

100 tests^[38]. The sensitivity of the test needs to improve as in a study the KAtex test result was positive in 87% of a large sample, the largest evaluated to date, a sensitivity > 95% is ideal for a diagnostic test^[39].

1.1.6.3 Lateral Flow test – rK39 RDT

This dipstick style lateral flow test which can provide a result using a drop of blood collected from a patient and provide results in around 10 min. Fig 7 below provides an overview to how the test is run.

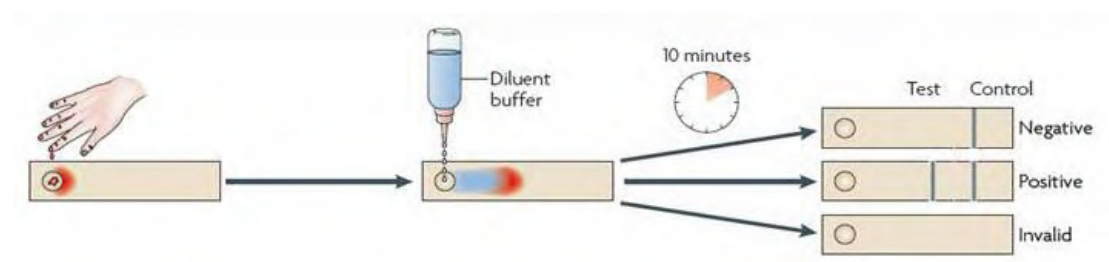


Fig 7 – rK39 dipstick test^[35]

These tests are very simple to run and only require a fresh drop of blood collected from a whole blood sample and a few drops of the buffer solution to give a result. The testing reagent is thought to be antibodies that recognise other antibodies in the blood sample, these are already present on the strip so no addition of reagents is necessary. Through capillary action the sample passes along the strip where it comes into contact with the testing antibodies that bind any parasitic antibodies found in

the blood. The test gives the results as either positive or negative. These tests are supplied individually wrapped, can be stored at room temperature and need no other equipment^[38]. By looking for specific antibodies against the parasite; this is very good in patients with an active infection. The problem is that once the infection has been treated and cleared, the body continues to produce anti-bodies against the past infection. Due to the continual expression of antibodies after the infection has past (around 3-4 months after) this test will continue to read positive for an infection^[40].

What is needed is a test-kit that brings the robustness of the rK39 RDT test, with detection of an active infection allowing treatment to stop when the infection stops. So the test would need to detect something that is parasite specific, and only present when the parasite is active in the patient.

1.1.7 Leishmania surface coat

The surface coat of the *Leishmania* parasite is very complex and is very important in the survival and infectivity in both the insect vector and mammalian host. Many of the major cell-surface macromolecules found on the surface are anchored to the plasma membrane by glycosylphosphatidylinositol (GPI) anchors. Glycoconjugates expressed by the *Leishmania* parasite during the promastigote stage, are seen in Fig 8 [41] [42] [43].

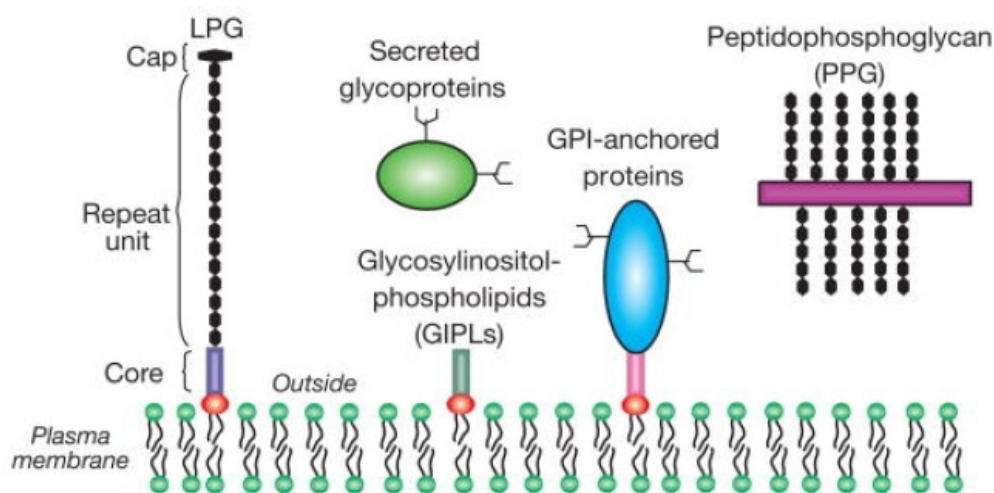


Fig 8 – Representation of the major cell-surface glycoconjugates of *Leishmania* [44]

The lipophosphoglycan (LPG) is a complex glycolipid forming a dense layer on the cell surface. The dense layer is made up with around 6×10^6 copies per promastigote cell. The LPG structure is made up of a GPI anchor linked to a phosphoglycan backbone of a repeat unit made up of $\text{Gal}\beta 1\text{-4Man}\alpha 1\text{-PO}_3\text{H}$ known as the phosphoglycan repeats (PG).

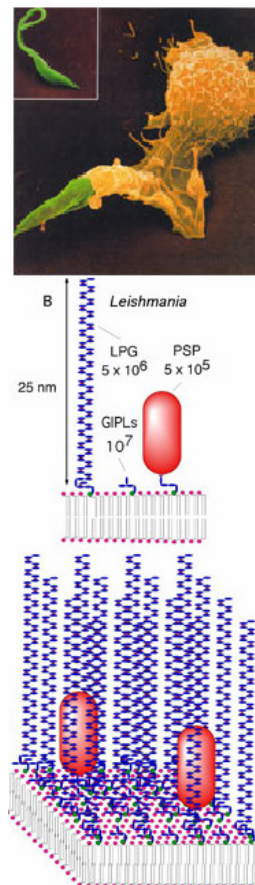


Figure 9 – *Leishmania* surface coat ^[45]

Glycoproteins such as GP46 ^[46], promastigote surface protease GP63 ^[47] and secreted forms of proteophosphoglycans ^[48] and finally a family of heterogeneous class of structurally related densely packed low-molecular mass glycoinositolphospholipids (GIPLs) can be found on the surface ^{[41][42]}.

1.1.8 The Structure of the Lipophosphoglycan (LPG)

The LPG has 3 main parts to its structure; the first is a phosphoinositol lipid anchor which binds to the cell surface holding the entire structure in place. Bound to this is a phosphosaccharide core which is also bound to the second important structure, the repeat domain, which is made up of the repeat unit $\text{Gal}\beta 1\text{-4Man}\alpha 1\text{-PO}_3\text{H}$. The final part is an oligosaccharide cap to terminate the chain^[42]. The anchor and phosphosaccharide core are much conserved in the *Leishmania* parasite. There are conserved areas in the repeat unit structure (the backbone) but it does contain some very species specific and life cycle specific variations. This can be clearly seen in Fig 10.

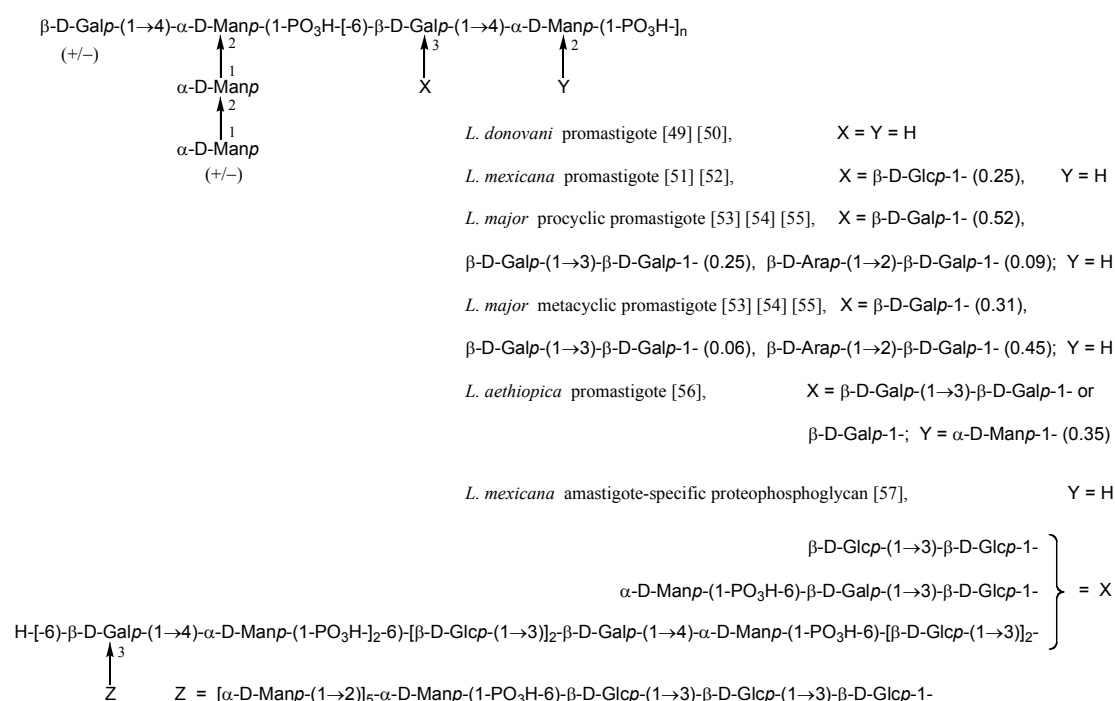


Fig 10 - LPG differences by species

The different species of *Leishmania* alter their LPG structure by adding branched sugars at different points, but always conserving the repeat unit backbone.

1.1.9 The role of the lipophosphoglycan

The characteristics and role of the LPG have been discussed extensively in reviews [58][59]. The LPG is associated with many important steps in the *Leishmania* infectious life cycle of both the vector and host [41][42][43]. It has been seen in knock out studies (knocking out genes used in the biosynthesis of the LPG) that the LPG is needed for the survivability of the parasite [21][58][59]. This makes the area of LPG biosynthesis an area of great interest.

1.1.10 The biosynthesis of the lipophosphoglycan repeat unit.

The Gal β 1-4Man α 1-PO₃H repeat unit which makes the backbone of the LPG structure is made by sequential addition of mannosylphosphate and galactose^[60] performed by a mannosylphosphate transferase (MPT) and a galactosyltransferase (GT) as seen in Fig 11.

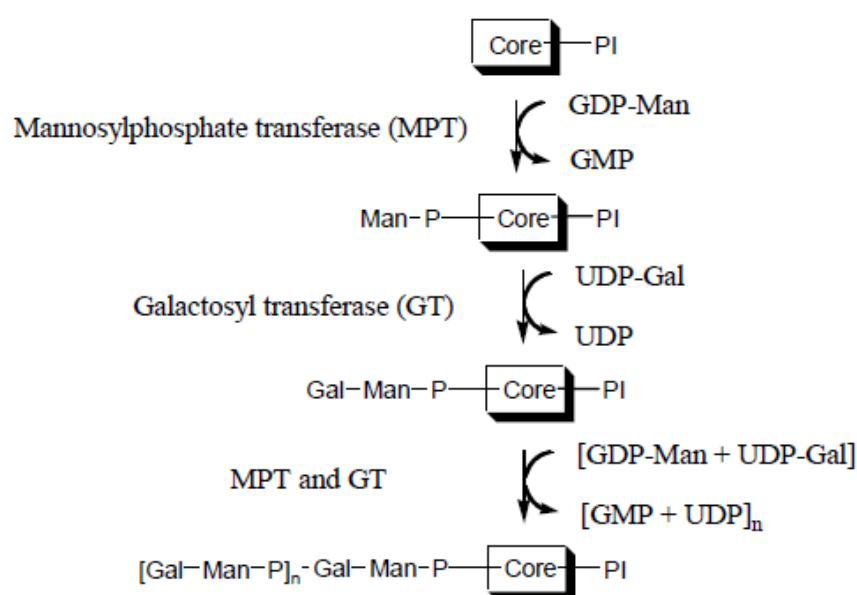


Figure 11 – Biosynthesis of LPG repeat domain

The repeat unit structure is not specific to the LPG; it is a structural component in many other glycoconjugates such as secreted and membrane proteins including sAP and PPGs. Since the repeat unit is used in many structures unique to the parasite including the extra-cellular LPG and secreted PPG's, it was thought that these repeat unit structures might be detectable. If it is possible to detect these structures then it would give a valuable parasite specific biomarker.

1.1.11 Detection of phosphoglycan repeats

In the lab of Marcel Hommel in the Liverpool School of Tropical Medicine a low molecular weight antigen (LMWA) was identified in the urine of visceral leishmaniasis patients^[61]. The methods they employed included; Capture ELISA, enzyme detection, trichloroacetic acid treatment, ethanol and acetone precipitation, Periodate treatment, Phenol-water extraction, Lipid extraction, Biotinylation, Affinity transfer blotting, Triton X-114 phase separation, Purification of GIPL's and finally detection by Leishmania anti-glyconjugate monoclonal antibodies. Unfortunately the group were unable to determine the structure of this LMWA they found, all they could say was that it was carbohydrate in nature and had very good binding with monoclonal antibodies raised against the LPG^{[62][63]}. This meant the structure found in the urine of the infected patient was some form of phosphoglycan repeat structure fragments from either the LPG or a PPG^[63a].

This was further confirmed by Ferguson's group in Dundee who were able to identify the phosphoglycan repeat structures by electrospray mass-spectrometry^[64]. The methods used were able to confirm the structure of the LPG fragments and could even detect any branched sugars from the various parasites. This method will enable us to type the LPG's and PG's in the biological samples such as urine.

1.1.12 A Possible New Test-kit for Leishmaniasis.

With the results talked about in the previous section it is now possible to think about a new diagnostic test-kit for leishmaniasis^{[38][39][40]}. The main problem with the rK39 dipstick test was that it could not detect when an infection had cleared because it detected the antibodies against the parasite. The new test looks for the antigen from parasite, this means when the parasite is gone (the patient is uninfected) there will be a negative result meaning treatment can halt. The urine of the patient will be tested, thus avoiding having to take blood from patients who live in areas with very high HIV+ infection rates.

By maintaining the idea of a lateral flow test (dipstick)(Fig 12 and 13) we can design a test using the phosphoglycans from the parasite and the antibodies to recognise them.

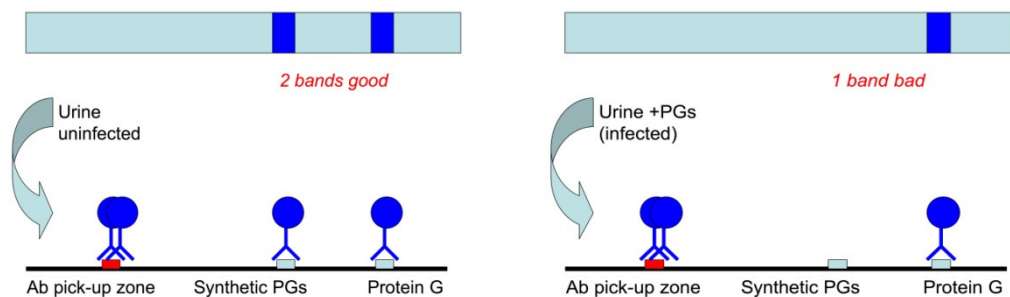


Figure 12 & 13 – lateral flow test ideas.

In this test, the urine (infected or not) is placed at the start of the strip and is immediately mixed with the anti-phosphoglycan antibodies bound to a marker. For an infected sample it is expected any phosphoglycans (PGs) in the urine will be picked up by the antibodies. Through capillary action the urine/antibody mixture is washed along the plate where it comes across synthetic PGs. For an infected sample there should be no binding with the synthetic PGs due to all antibodies being blocked by PGs found in the urine. For an uninfected sample there should be binding with the synthetic PGs. The capillary action continues to move the sample to end point where the test is complete. For an uninfected patient there would be 2 bands, one to show binding with the synthetic PGs and another to show excess antibodies at the end (Fig 12). For an infected patient there would be one band, at the end, as there should be no binding with the synthetic PGs due to all antibodies being blocked by PGs found in the urine.

For this test to work synthetic phosphoglycans are needed, thus providing the positive and negative comparison. They must be designed so they are recognised as the repeat unit structure and they must contain a region that can be bound to a solid surface for testing. A selection of repeat unit lengths (**1 - 3**) should be synthesised (Fig 14). Once synthesised, the compounds will undergo ELISA experiments to measure the binding of the monoclonal antibodies to establish how many repeat units are needed. Once this is complete the next steps will involve comparing the binding to antibodies and urine samples of infected patients. The synthesis of these compounds makes up the core topic of this thesis.

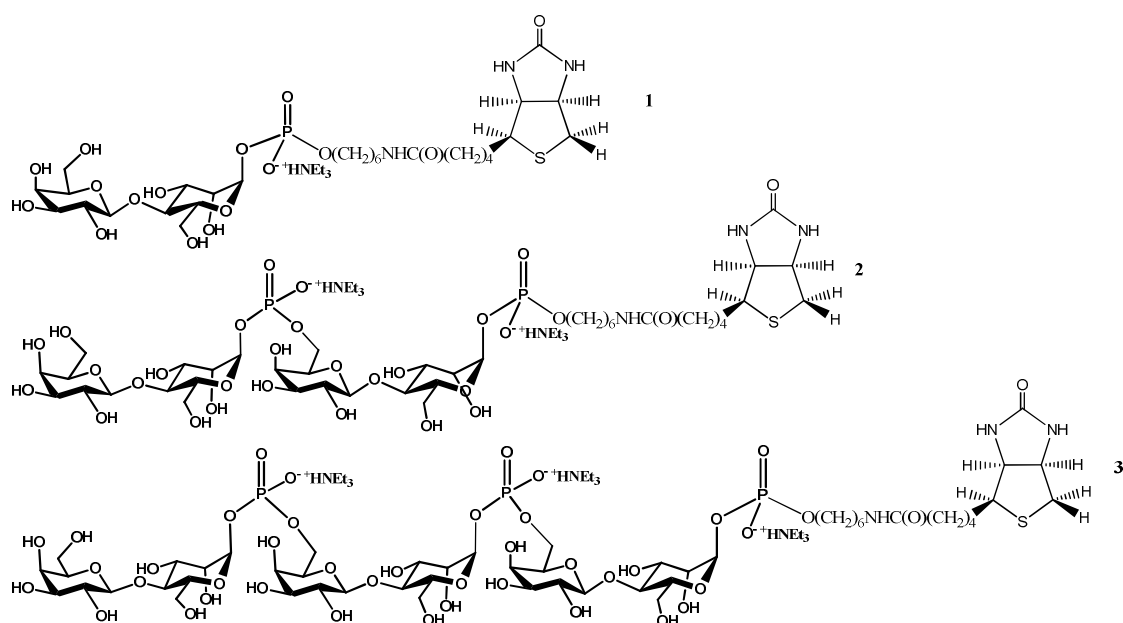


Figure 14 – Synthetic phosphoglycan repeats linked to biotin moiety

1.2 Chemical Introduction

1.2.1 Chemical synthesis of *Leishmania* LPG repeats

The development of synthetic approaches to phosphoglycans in general and *Leishmania* LPG in particular is not an easy task because of the hydrolytic lability of the phosphodiester bridges, which link the repeating units through the anomeric OH of D-mannose and the 6-OH of D-galactose. Wide-ranging reviews of *Leishmania* phosphoglycans have been published.^{[65][66][67]} These reviews cover a variety of approaches, stepwise, blockwise, polymer-supported and polycondensation, for the chemical synthesis of phosphoglycans.

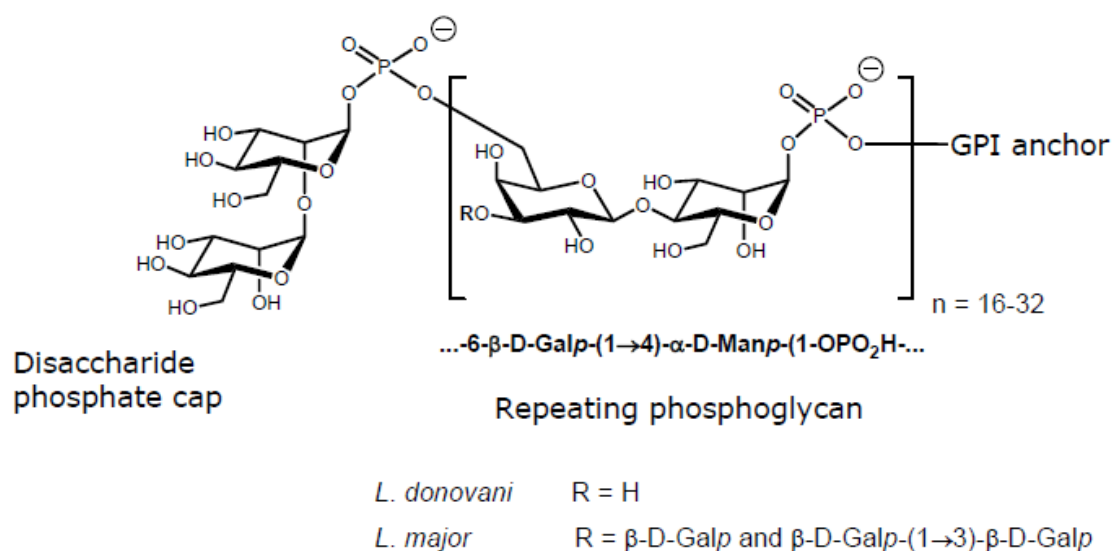
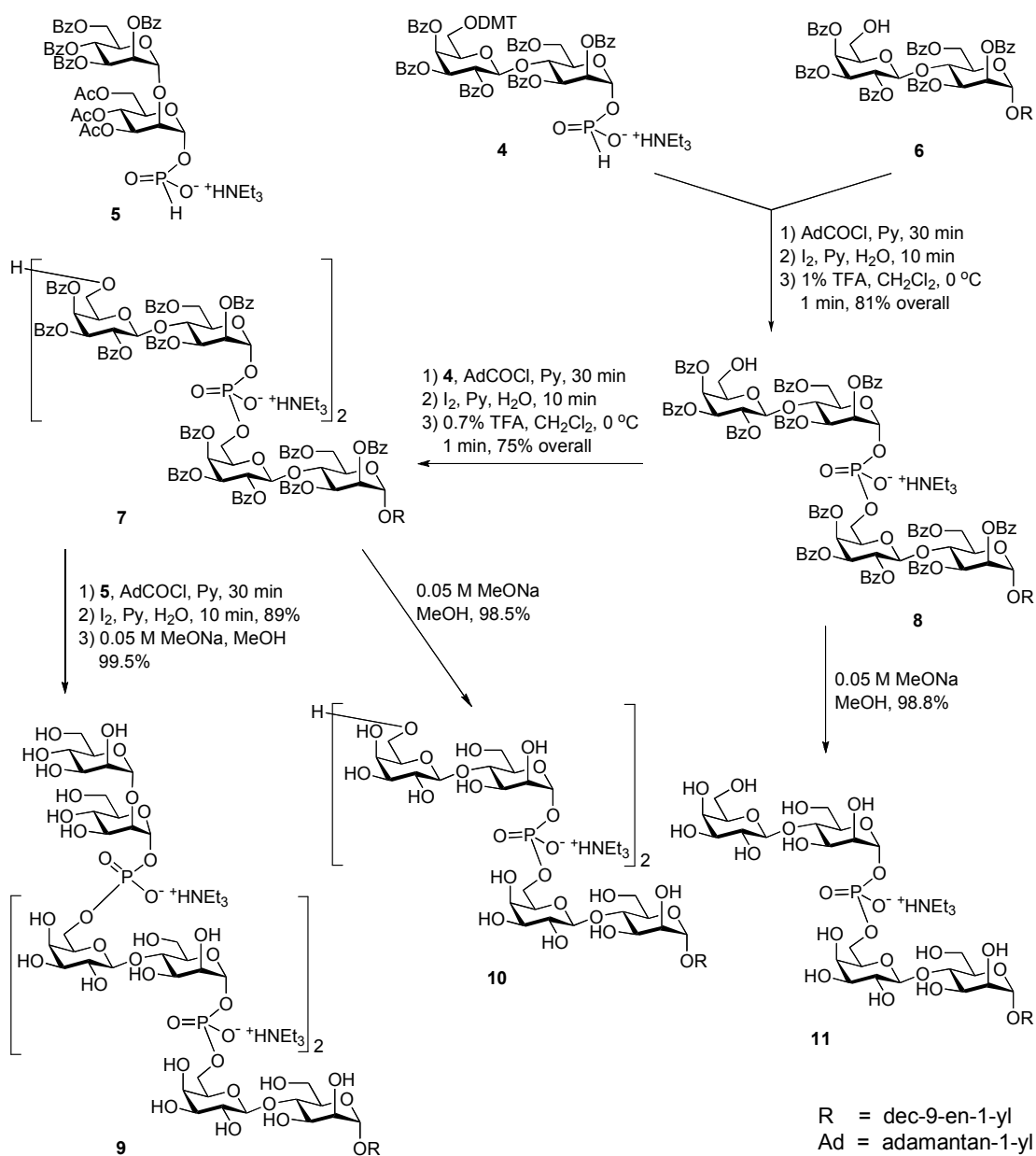


Fig 15 – Structure of the LPG of *L. donovani* and *L. major*

As described before the LPG domain contains the oligosaccharide cap, the repeat domain and the GPI anchor. The size of the repeat domain depends on the parasites stage of development. Many groups have been working on the synthesis of the LPG concentrating on different areas. The Vishwakarma^[68], Fraser-Reid^[69] and Seeberger^{[70][71]} groups synthesised the LPG cap domain. Synthesis of the *Leishmania* LPG core has been performed jointly by two groups: Oscarson and co-workers^[72] and Konradsson and co-workers^{[73][74]}. The LPG repeat domain has been worked on by two main groups, Nikolaev's group from University of Dundee and Vishwakarma's group from New Delhi. Both have attempted synthesis of the repeat units using different synthetic strategies. Since the important synthetic aim of the project is to produce repeat unit structures the different approaches will be discussed.

1.2.2 Nikolaev and co-workers

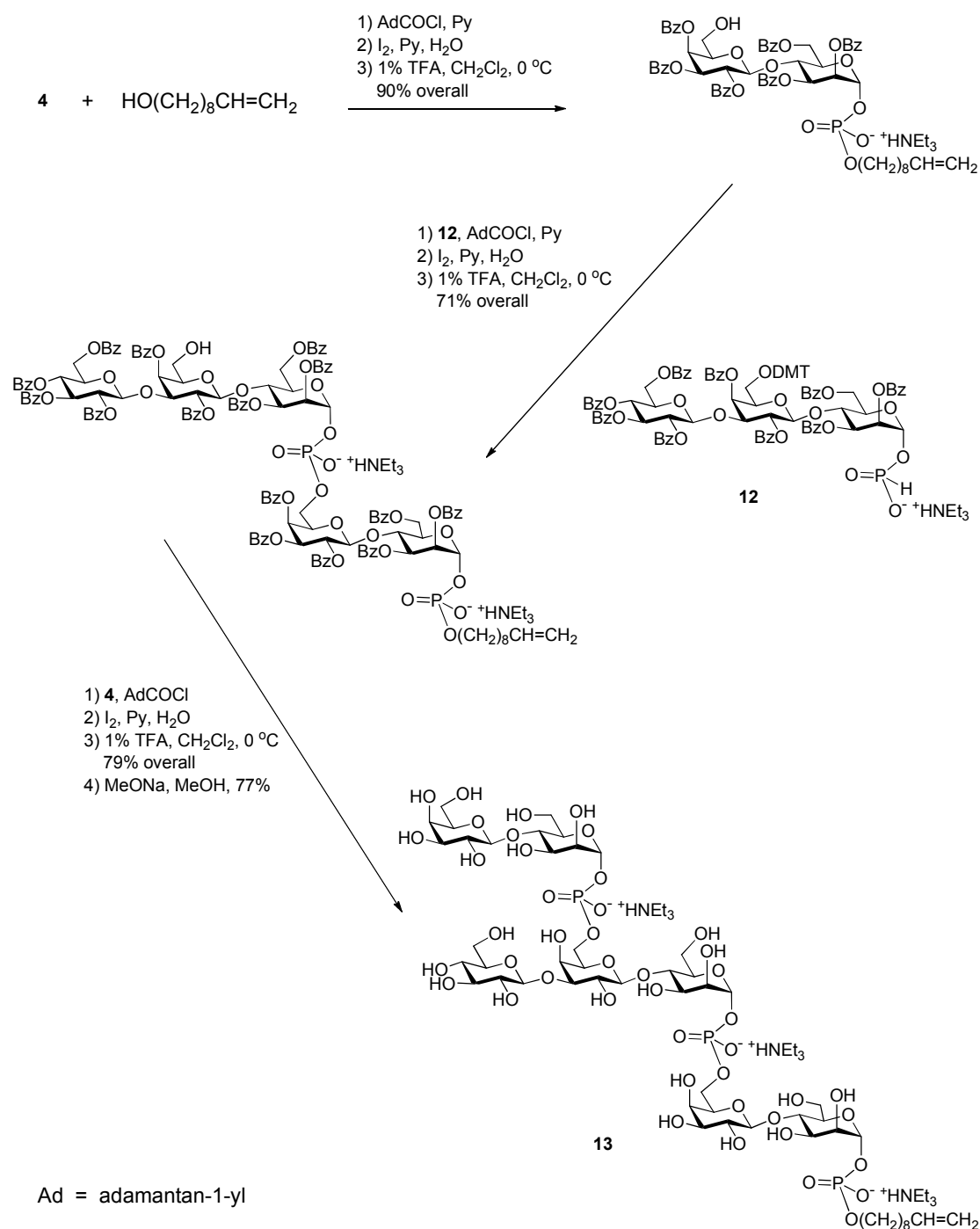
The first successful syntheses of the *Leishmania* phosphoglycans were achieved by the Dundee University based group^{[75][76]} by exploiting H-phosphonate chemistry. Several successful syntheses of *L. donovani* phosphoglycans containing 2 and 3 repeat units and with the cap unit attached were synthesised (Scheme 1) using the stepwise approach to chain elongation. The glycosyl H-phosphonate route was proven to be the method of choice for the efficient and reliable assembly of various phosphodiester linkages^[77].



Scheme 1 –Stepwise synthesis of phosphoglycans of *L. donovani*.^[78]

Protected tetrasaccharide phosphate **8** was synthesised by the coupling of the disaccharide H-phosphonate **4** and the disaccharide **6** with one free hydroxyl group, followed by the removing of the DMT protecting group. Addition of another H-

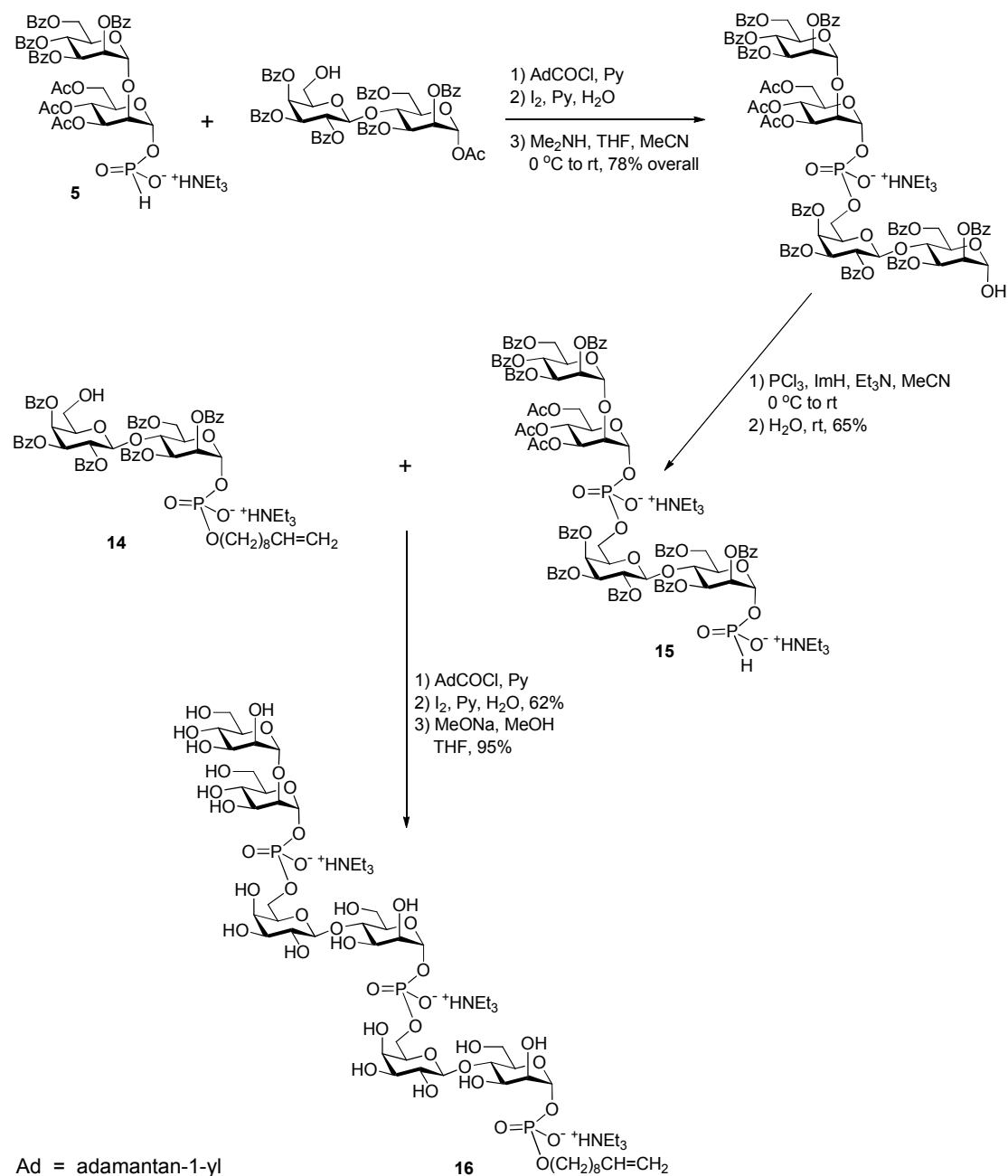
phosphonate **4** and subsequent oxidation yielded the hexasaccharide diphosphate **7** after de-*O*-tritylation. Further chain extension using the disaccharide H-phosphonate **5** and compounds **7** followed by global deprotection gave the octasaccharide phosphoglycan **9**. Protected fragments **7** and **8** also underwent global deprotection giving compounds **10** and **11**, respectively.



Scheme 2 – Stepwise strategy to *L. mexicana* LPG

Using the stepwise addition of H-phosphonate units **4** and **12** the branched phosphoglycan of *L. mexicana* **13** was synthesised (Scheme 2)^[79]. In addition, several LPG fragments from *L. major* were also prepared.^{[80][81][82][83][84]}

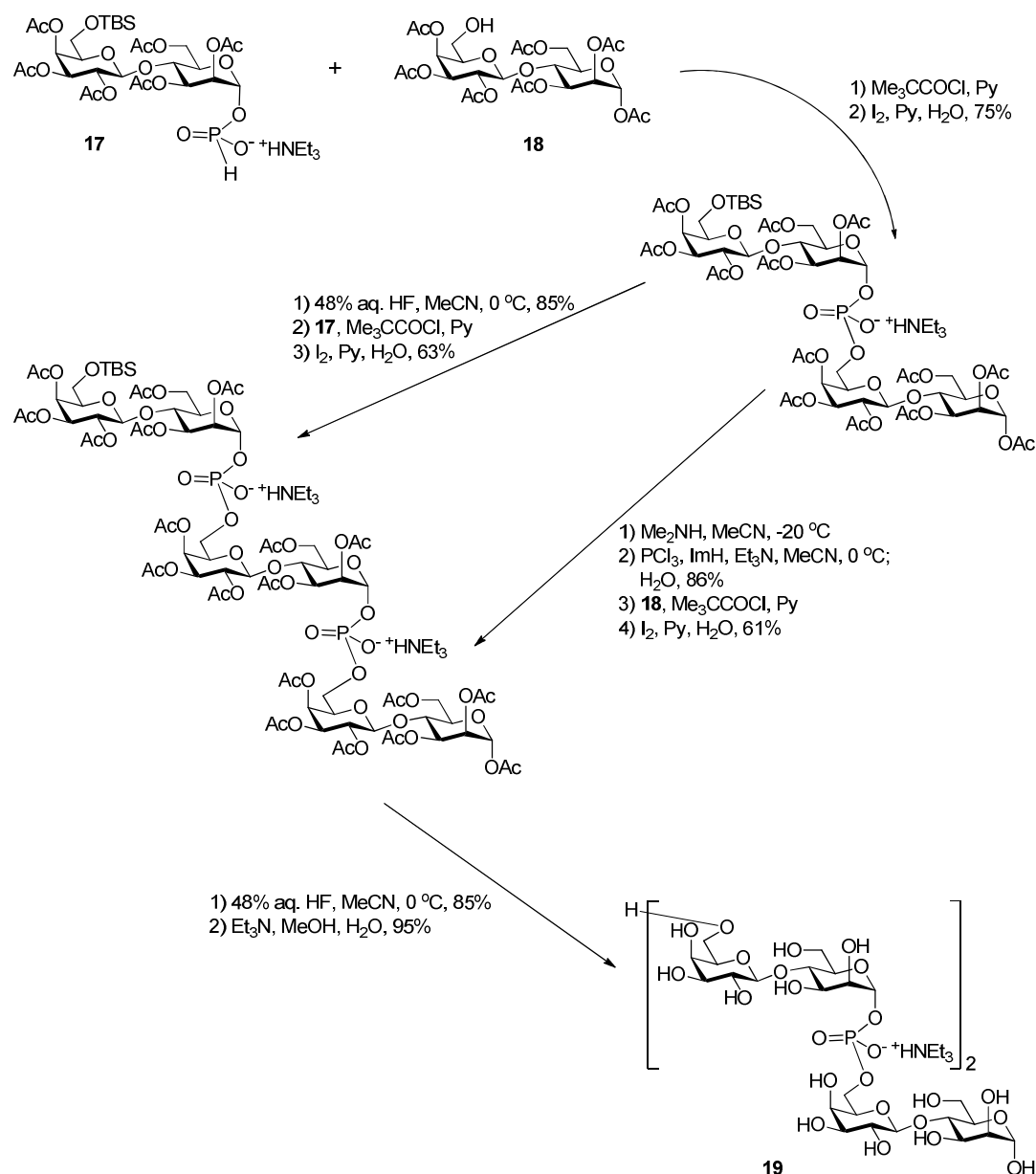
A blockwise approach was also developed by Nikolaev *et al.* [85] this involves the condensation of two phosphodiester blocks: the H-phosphonate derivative **15** and 6'-hydroxyl acceptor **14** for the synthesis of the hexaglycosyl triphosphate **16**, which is a terminal fragment of the phosphoglycan portion of *L. donovani* LPG (Scheme 3).



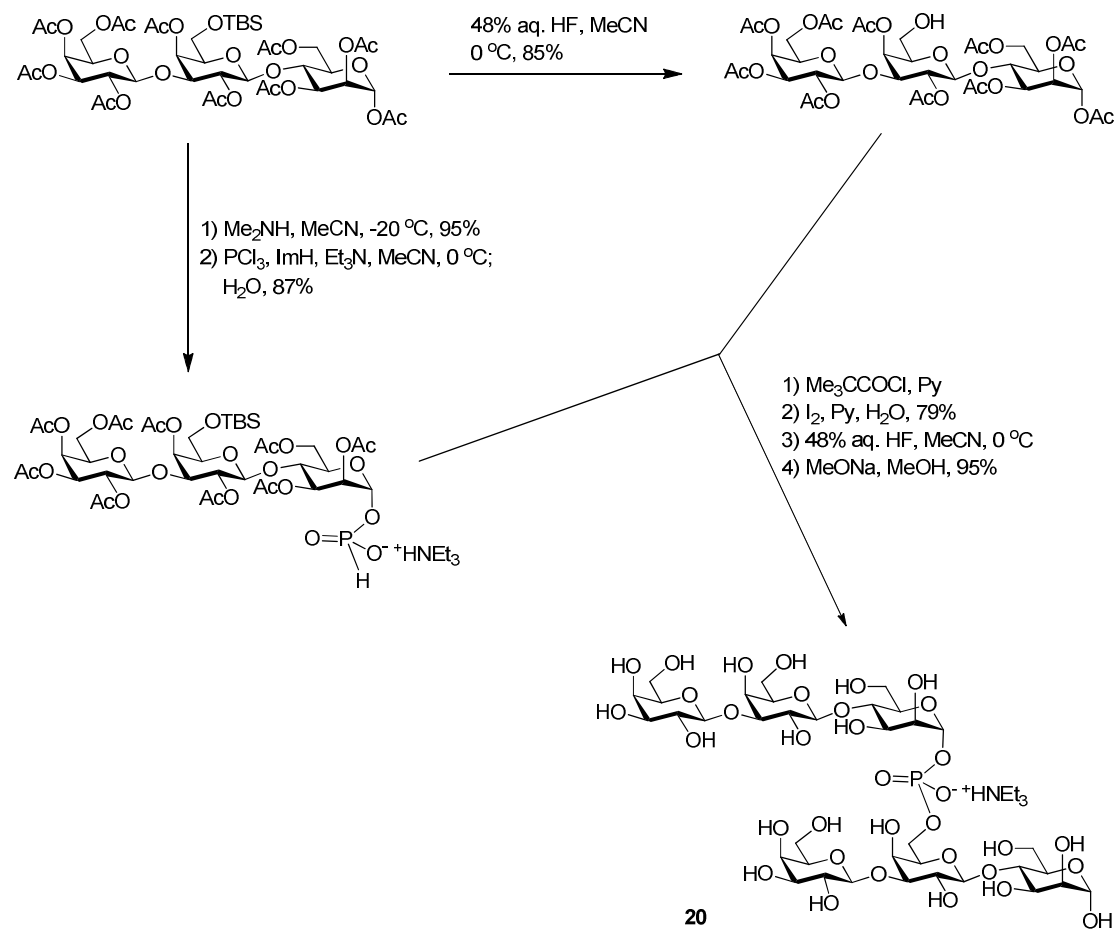
Scheme 3 –Blockwise strategy to *L. donovani* phosphoglycan

1.2.3 Vishwakarma and co-workers

Vishwakarma and co-workers also described an iterative synthesis of the *L. donovani* phosphoglycan **19** (Scheme 4).^{[86][87]} as well as the branched phosphoglycan **20** of *L. major* (Scheme 5)^[88] using traditional solution phase chemistry.



Scheme 4 – The preparation of *L. donovani* phosphoglycans



Scheme 5 - The preparation of the branched phosphoglycan of *L. major*.

1.2.4 Anchor and Spacer Selection

The anchor part of the synthetic phosphoglycans **1-3** (Fig 14) must be stable during the chemical synthesis and when needed bind to a solid surface. The anchor that was chosen was biotin (Fig 16); vitamin H or Coenzyme R has been used for many biological applications. By biotinylating unknown proteins or compounds in solution they can be anchored and identified. The anchoring occurs with the use of avidin proteins which bind biotin with a very high affinity ($K_d \sim 10^{-15}$ mol/L). This provides the biochemists with the ability to capture unique compounds from solution.

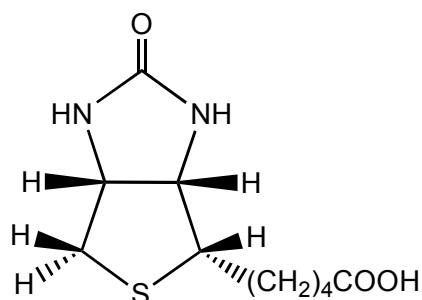


Figure 16 – Biotin

Biotin will be incorporated into the synthetic phosphoglycans with a spacer molecule which will be 6-aminohexanol. The spacer is used just to separate the phosphoglycan repeats and the biotin. There has not been a great deal of work from a synthetic carbohydrate chemistry perspective using biotin but a paper modifying biotin ^[89] was published from the laboratory of Nikolaev and will form the basis of chemistry involving biotin.

2. RESULTS AND DISCUSSION

2.1. Aims of the project

A biomarker for the *Leishmania* parasite has been identified in the lab of Mike Ferguson in Dundee. It was found that this biomarker is a ...-6-β-D-Galp-(1-4)-α-D-Manp-(1-OPO₂H- ... (Fig 17) repeat unit of the repeat domain of the lipophosphoglycan (LPG), the most abundant macromolecule on the surface of promastigote forms of all *Leishmania* species.

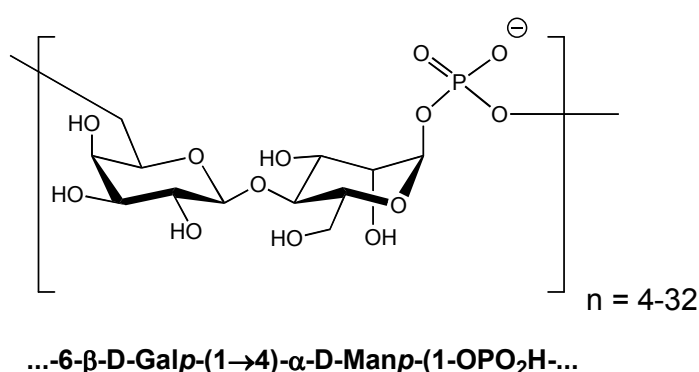


Fig 17- Repeat domain and the repeat unit of *Leishmania* LPG

This repeat unit was found to be detectable in the urine of an infected patient, and since this phosphoglycan structure is parasite specific it gives a very good basis for a diagnostic test. Furthermore, since this phosphoglycan is parasite specific once the infection has been treated there will be no biomarker present in the patient. In further work monoclonal antibodies were identified that recognise this structure and could be used as part of the test kit. So, the idea of a test kit was put together (Fig 18) where the urine of

an infected patient would be tested with the antibodies to give a result either positive for an active infection (Fig 18) or negative for no infection (Fig 19).

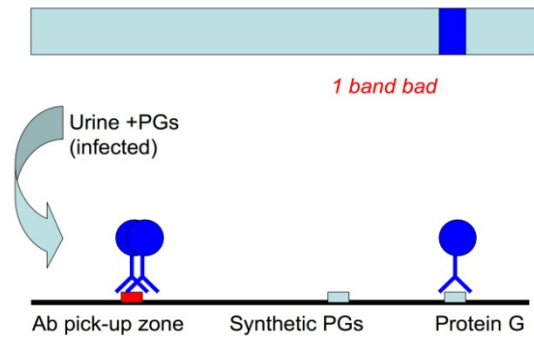


Fig 18 – Positive test

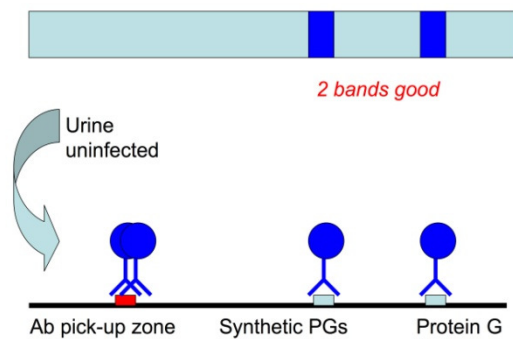


Fig 19 – Negative test

With this style of dipstick test a positive control is needed, so synthetic compounds of the repeat unit must be synthesised. The repeats must be recognised by the antibody and provide detectable binding to provide a result. These repeats must also be able to bind with a surface on which the testing can occur.

Three target compounds **1-3** (Fig 20) were decided upon of different repeat unit lengths, all attach to a biotin moiety that can be used to anchor the compounds to an avidin-coated surface.

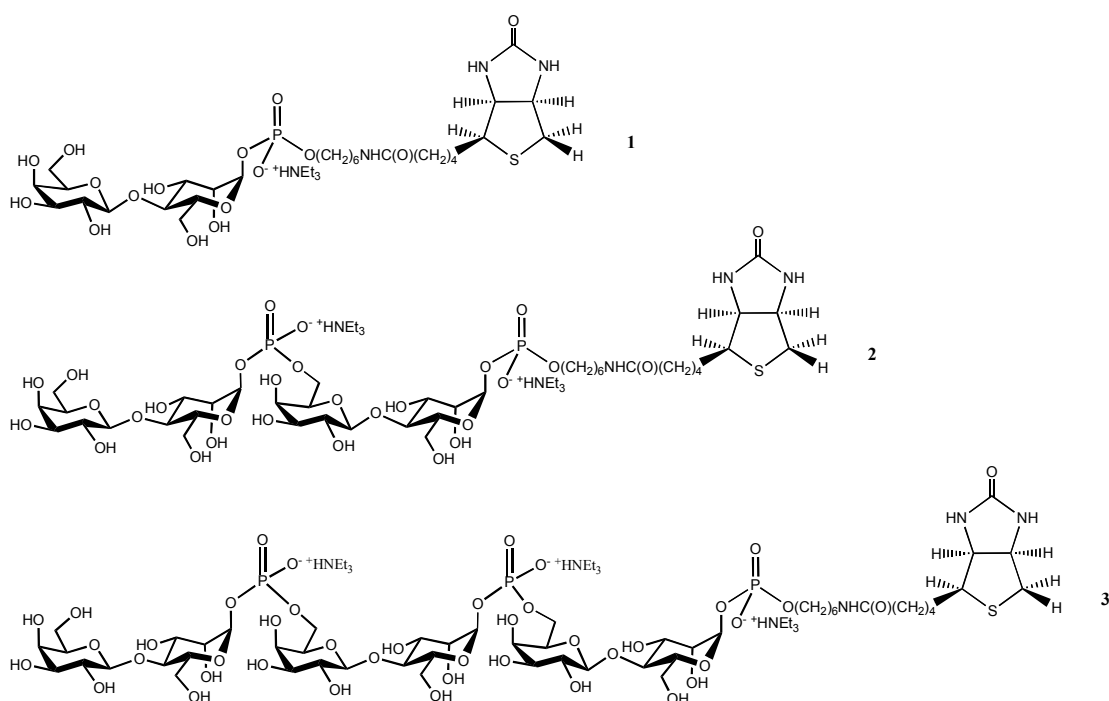


Fig 20 – Original target compounds: biotinylated LPG fragments **1-3**

After the synthesis of the compounds they will undergo biological testing in an ELISA assay to measure the binding with the antibody to establish which compound length is most suitable.

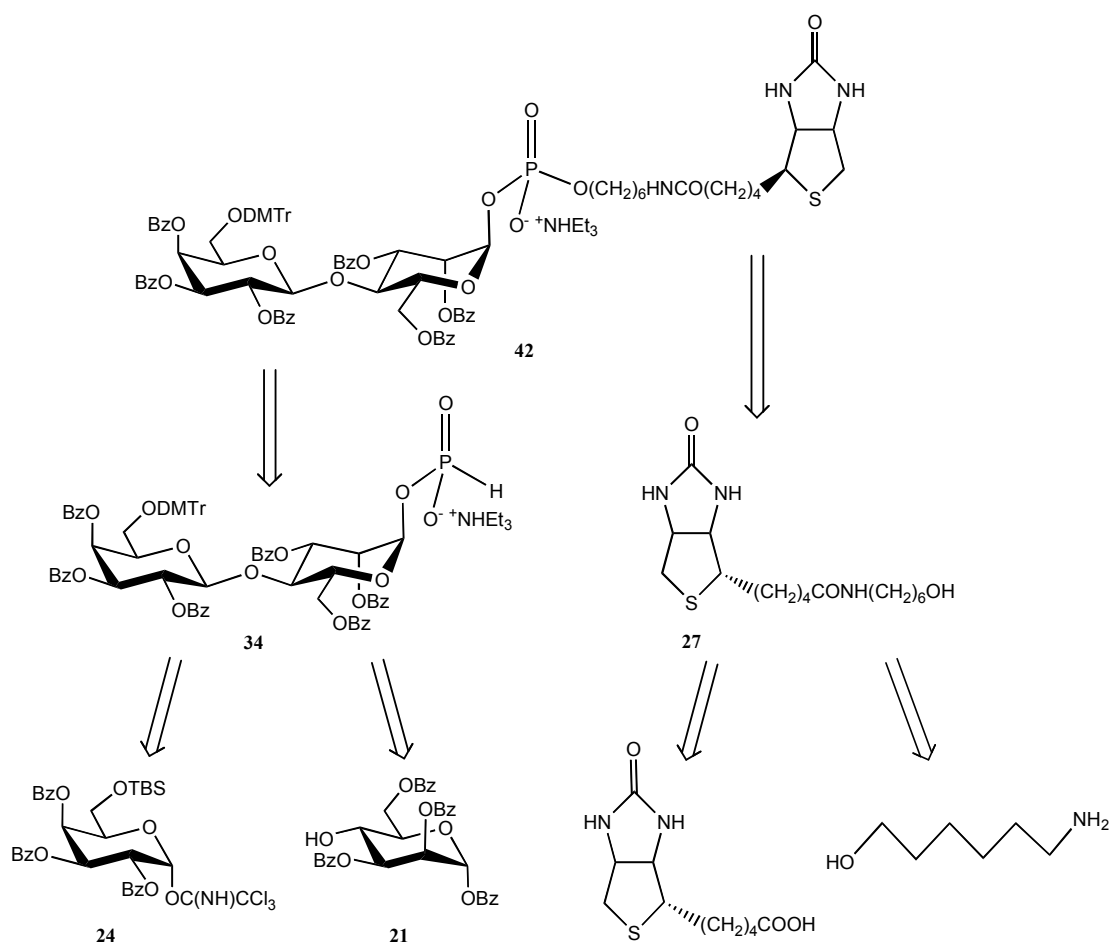
2.2. General synthetic design

The chemical preparation of compounds **1-3** was attempted and appeared to be unsuccessful (see Section 2.3.7), although an alternative strategy was developed later (see Section 2.5), which was successful for the preparation of different structures containing one, two and three repeat units connected to a biotin moiety. This was completed using the synthetic schemes which contained a few general steps:

- 1) Preparation of selectively protected monosaccharide and disaccharide derivatives.
- 2) Preparation of protected H-phosphonate derivatives.
- 3) Preparation of protected phosphoglycan structures using the glycosyl H-phosphonate condensation method (including biotin addition).
- 4) Total deprotection through de-*O*-benzoylation.

2.3 Synthesis of 6-*N*-Biotinylaminohexyl 2,3,6-tri-*O*-benzoyl-4-*O*-[2,3,4-tri-*O*-benzoyl-6-*O*-(*p,p'*-dimethoxytrityl)- β -D-galactopyranosyl]- α -D-mannopyranosyl phosphate, triethylammonium salt **42**

A retrosynthetic scheme that enables the synthesis of the target compounds has been identified (Scheme 8). The building blocks required for the compounds **1-3** are a modified (with 6-aminohexanol linker) biotin derivative **27** and the repeat unit β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranosyl H-phosphonate derivative **34**.

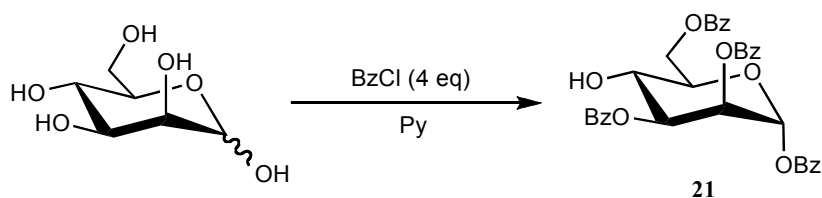


Scheme 8 - Retrosynthetic strategy to the disaccharide phosphate **42**, which is a protected derivative of the target compound **1**

For the additional target compounds **2** and **3** extra repeat units will be added through the chain elongation using the protected disaccharide H-phosphonate derivative **34**. This was to be done after the incorporation of the biotin derivative to the first repeat.

2.3.1 Synthesis of 1,2,3,6-Tetra-*O*-benzoyl- α -D-mannopyranose **21**

The glycosyl acceptor **21** (Scheme 9) for the disaccharide synthesis is a α -D-mannopyranose derivative protected at positions 1,2,3 & 6 with a benzoyl protecting group leaving the hydroxyl at position four free^[91]. This can be done by selective benzylation using a dropwise addition of a strict 4 eq. of benzoyl chloride to a solution of D-mannose in pyridine at -40 °C.



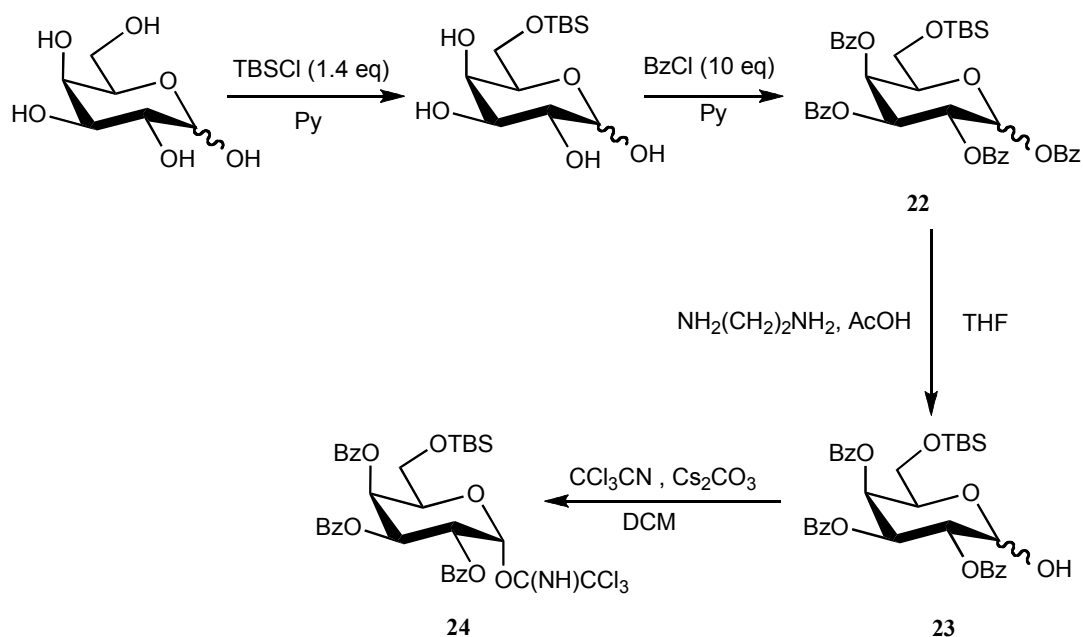
Scheme 9 – Preparation of the glycosyl acceptor **21**

Use of a strict 4 eq. of the reagent means the benzoyl chloride will react with the most reactive HO-groups first leaving the least reactive unprotected. In the case of D-mannose 4-OH is the least reactive group giving the required selectivity. Derivative **21** was

recrystallised from ethyl acetate and *n*-hexane giving crystals with a melting point of 184-185 °C.

2.3.2 Synthesis of 2,3,4-Tri-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl- α -D-galactopyranosyl trichloroacetimidate **24**

The D-galactopyranosyl trichloroacetimidate **24** (Scheme 10) was synthesised starting from D-galactose.



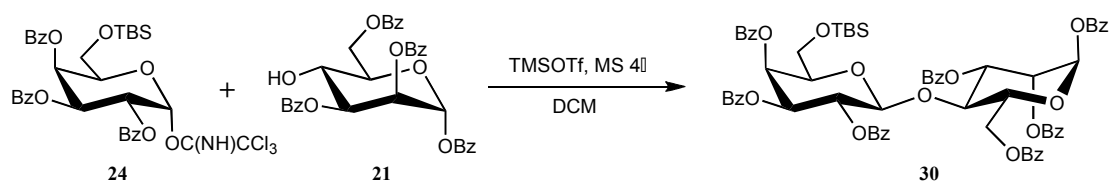
Scheme 10 – Preparation of the glycosyl donor **24**

D-Galactose was firstly reacted with *tert*-butyldimethylsilyl chloride in pyridine for 2 hours. This provided a silyl protecting group at position 6, which would become important in further steps. Once the silyl protection was in place, benzoyl chloride (10 eq) was

added to protect the rest of the free hydroxyls of D-galactose. After a work-up and purification on silica gel this gave a fully protected galactose derivative **22** in a good yield of 82 %. The next step required the formation of the hemiacetal by removing the benzoate from the anomeric position. This was done using two methods, the first used dimethylamine (2M) in THF and this method took around 12 hours and gave a yield of 59% ^{[90][91]}. The second method took considerably longer at 30 hours but the yield was higher at 75%; this method used ethylenediamine and acetic acid in THF^[92]. Both methods were used during the repeated syntheses of the hemiacetal **23**. Formation of the glycosyl donor **24** from the hemiacetal was done using trichloroacetonitrile and a catalytic amount of cesium carbonate in DCM ^{[93][94]}. The reaction took around 1 hour to complete, with the yield of 64 %. The formation of the glycosyl trichloroacetimidate **24** was verified with by ¹H and ¹³C NMR. From the ¹H NMR the signal for the NH of the trichloroacetimidate at 8.69 ppm, can be clearly seen.

2.3.3 Synthesis of 1,2,3,6-Tetra-*O*-benzoyl-4-*O*-(2,3,4-tri-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl- β -D-galactopyranosyl)- α -D-mannopyranose **30**

With the synthesis of the glycosyl donor **24** and the glycosyl acceptor **21** the derivative to form is the protected disaccharide **30** (Scheme 11).

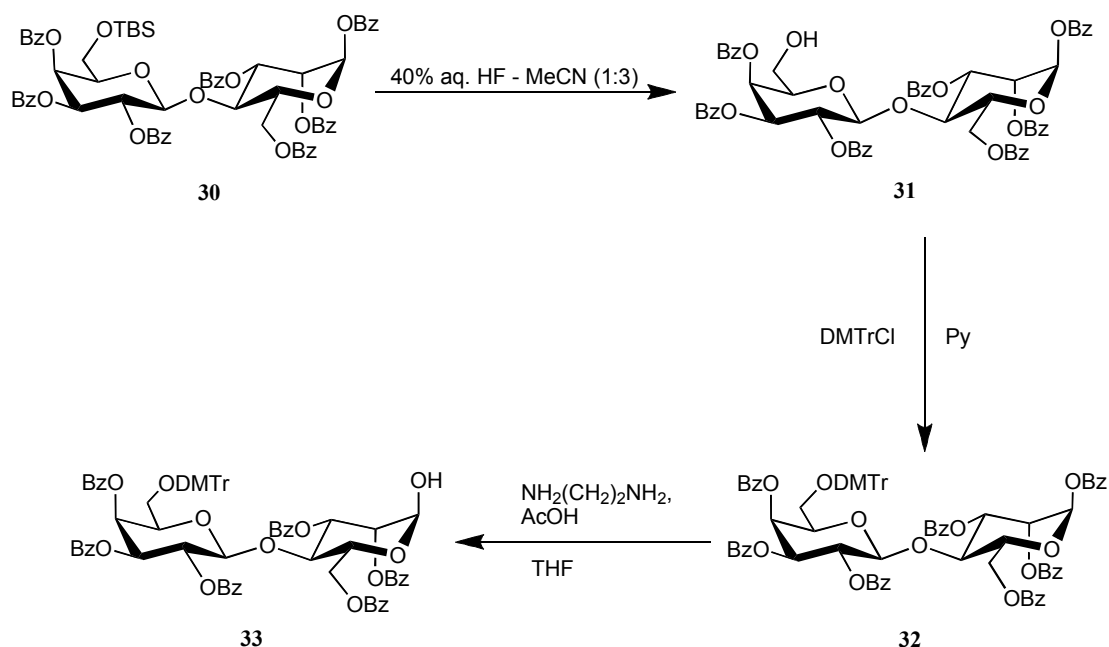


Scheme 11 – Preparation of the disaccharide **30**

The glycosylation of the 4-OH D-mannose acceptor **21** with the trichloroacetimidate galactose donor **24** was catalysed by Trimethylsilyl trifluoromethanesulfonate (TMSOTf), with molecular sieves 4Å to ensure anhydrous conditions^[95]. The reaction proceeded smoothly with the major product being the β -linked disaccharide 1,2,3,6-tetra-*O*-benzoyl-4-*O*-(2,3,4-tri-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl- β -D-galactopyranosyl)- α -D-mannopyranose **30** formed in a 72 % yield.

2.3.4. Synthesis of 2,3,6-Tri-*O*-benzoyl-4-*O*-[2,3,4-tri-*O*-benzoyl-6-*O*-(*p,p'*-dimethoxytrityl)- β -D-galactopyranosyl]- α -D-mannopyranose **33**

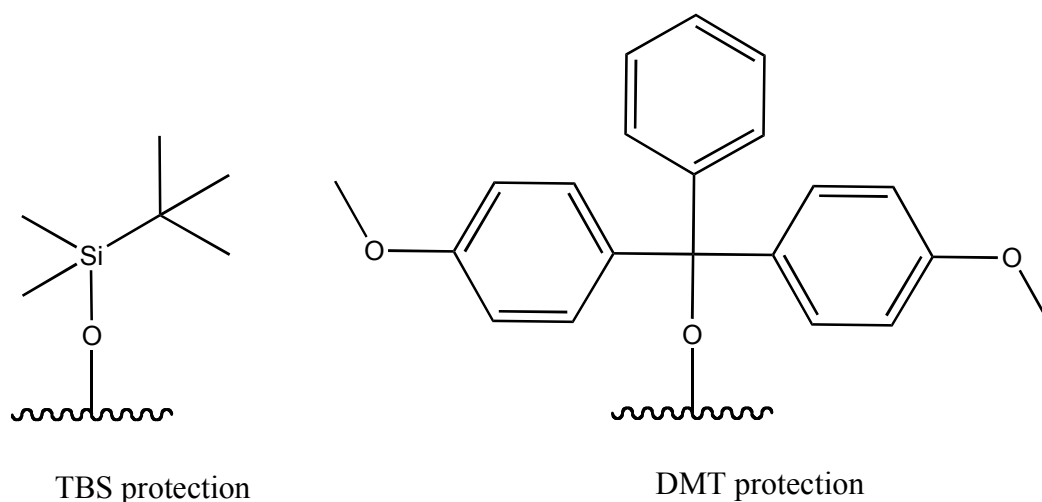
2.3.4.1 Method A



Scheme 12 – Preparation of the disaccharide **33**

From derivative **30** removal of the silyl protection group was carried out using a mixture of 40 % aq. HF and acetonitrile (1:3) at 0 °C^[96]. This removed the silyl group at position 6 and provided the disaccharide **31** (Scheme 12) in a 95 % yield. This was further proved by the complete disappearance of the *tert*-butyldimethylsilyl signals in the ¹H and ¹³C NMR of **31**. Compound **31** was then re-protected at position 6 of the D-galactose residue, this time with a *p,p'*-dimethoxytrityl protecting group. The reason for the replacement of the protecting group at this stage and not at the beginning of the synthesis of the

disaccharide **30** is due the very acid sensitive nature of the *p,p'*-dimethoxytrityl protection (which proved to be highly beneficial during further phosphosaccharide chain elongation).



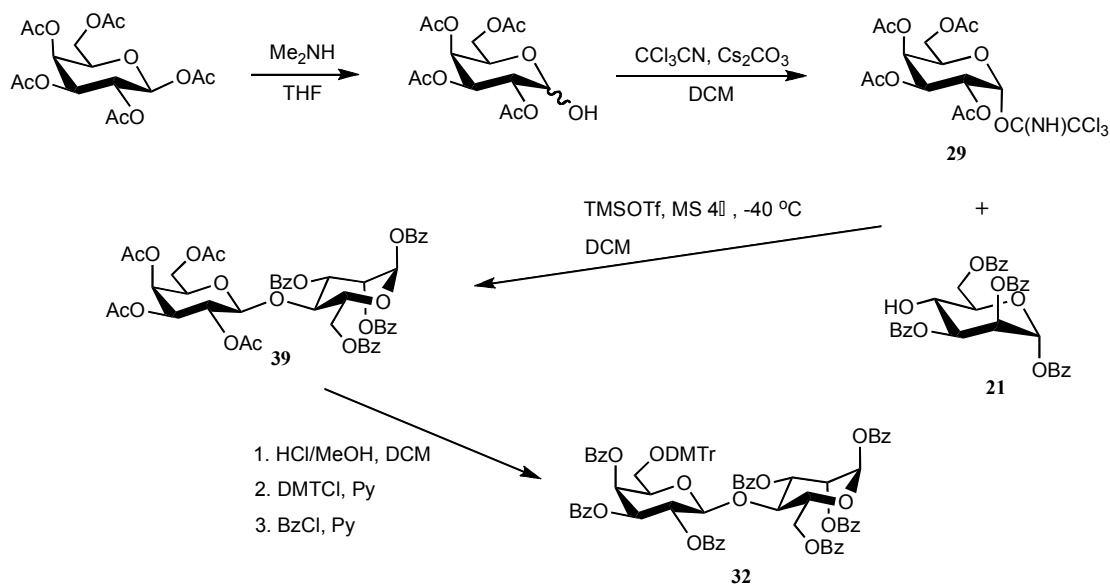
Scheme 12a – TBS and DMT protecting groups

If the *p,p'*-dimethoxytrityl group was present from the beginning of the synthesis during the previous step of glycosylation (see Section 2.3.3) it would have been cleaved. This would have resulted in two free hydroxyl groups during the glycosylation reaction resulting in many side products. By changing the TBS protecting group to the DMT (Scheme 12a) this gives fast effect removal, 2 min for *p,p'*-dimethoxytrityl compared with 3 h for silyl. In addition, the *p,p'*-dimethoxytrityl derivatives can be easily identified by the bright orange colour they give when detected on TLC plates with a mixture of sulphuric acid-water-ethanol (15:85:5). Tritylation of compound **31** was carried out with *p,p'*-dimethoxytriphenylmethyl chloride in pyridine. This took around 15 h to complete giving the derivative **32** in an 82% yield. The formation of the hemiacetal **33** was carried out

using the dimethylamine method ^{[90][91]} yielding 70% in 20 h and the ethylenediamine method ^[92] was complete in 40 h giving a yield of 75%. Dimethylamine was used as the method of choice in this step due to the lower reaction time. With the hemiacetal disaccharide **33** prepared in a good yield over the 4 steps (starting from monosaccharide compounds **21** and **24**), the building block for the H-phosphonate disaccharide repeat unit derivative, was now available

2.3.4.2 Method B

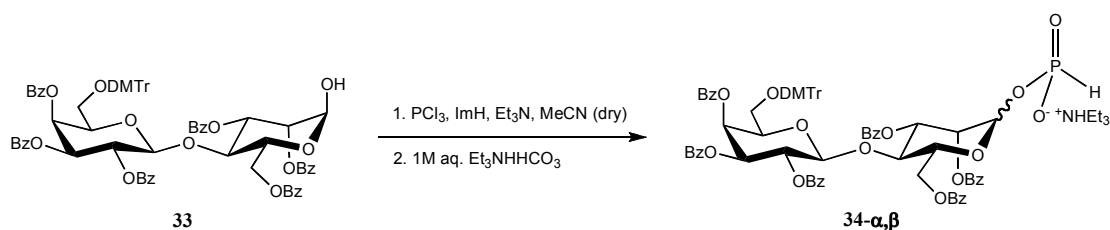
Another method for the formation of this DMT protected disaccharide **32** was designed. This method (Scheme 13) reduces the number of steps and purifications needed to synthesise this compound.^[93]



Scheme 13 – Alternative synthesis of the disaccharide **32**

The pentaacetate of β -D-galactose underwent anomeric deprotection with dimethylamine in THF.^{[90][91]} After 1 h it could be seen (monitoring by TLC) the reaction was complete, and the crude hemiacetal derivative was taken straight into the next step. The formation of the trichloroacetimidate **29** was carried out in DCM with trichloroacetonitrile and a catalytic amount of cesium carbonate^[97]. After 2 h the reaction was worked up and the product was purified. This gave the trichloroacetimidate **29** in a yield of 80% for the two steps. The glycosylation reaction between derivative **29** and the tetrabenzoate **21** was carried out with TMSOTf as the catalyst, and run for 30 min at -40 °C^{[95][97]}. Purification of the reaction products resulted in the disaccharide **39** in a yield of 88 %. The disaccharide **39** then underwent a deprotection step to remove all the acetate groups from the D-galactose residue, which was achieved by using HCl in MeOH. This method is highly selective to cleave *O*-acetyl protecting groups in the presence of benzoic esters^[98]. Once the de-*O*-acetylation was complete the reaction was quenched and worked-up. The next step was to dissolve the product in pyridine and add the *p,p'*-dimethoxytriphenylmethyl chloride; this was left for 20 h after which benzoyl chloride was added and the reaction mixture was left for a further 15 h^[97]. The reaction was then stopped and worked-up; after the purification the disaccharide **32** was isolated in a 91 % yield for the 3 steps.

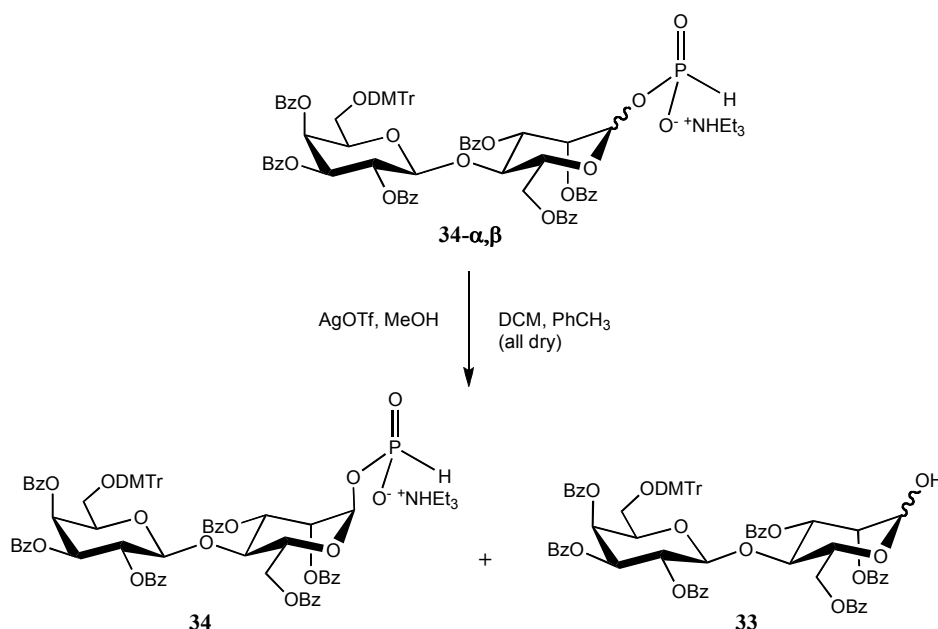
2.3.5 Synthesis of 2,3,6-Tri-*O*-benzoyl-4-*O*-[2,3,4-tri-*O*-benzoyl-6-*O*-(*p,p'*-dimethoxytrityl)- β -D-galactopyranosyl]- α -D-mannopyranosyl hydrogenphosphonate, triethylammonium salt **34**



Scheme 14 – Preparation of the disaccharide H-phosphonate with PCl_3 , imidazole and Et_3N

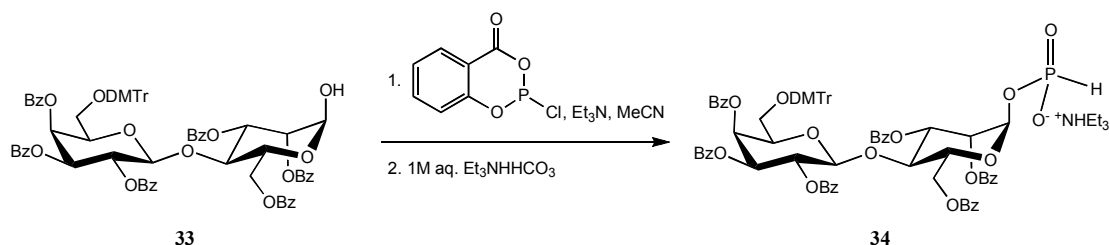
The synthesis of the H-phosphonate derivative **34** was achieved via the disaccharide hemiacetal **33** (Scheme 14). Phosphitylation of the hemiacetal was carried out using triimidazolylphosphine, which was prepared *in situ* from PCl_3 , imidazole and Et_3N , followed by hydrolysis with aq. TEAB buffer solution (pH 7.8)^{[99][100]}. This provided the glycosyl H-phosphonate derivative **34- α,β** in a very good 95 % yield, but as an anomeric mixture at the D-mannose residue with α/β ratio of 12:1. To proceed on in the synthetic strategy a ratio of >20:1 would be preferred to ensure the correct anomeric configuration. To improve the α/β ratio, the derivative **34- α,β** was reacted with silver triflate and methanol for 40 min at room temperature^[101]. This allowed degradation (solvolysis) of the β -H-phosphonate back to the original hemiacetal **33** (Scheme 15). The glycosyl β -H-phosphonate (with *equatorial* H-phosphonic moiety at C1) is known to be less stable than the corresponding α -anomer (with *axial* H-phosphonic moiety at C1), which is favoured

by the anomeric effect^[102]. It was seen by ³¹P NMR that there was no β-anomer present and the pure α-anomer **34** was isolated in a 50% yield. The hemiacetal **33** was recovered and used again to prepare the disaccharide H-phosphonate.



Scheme 15 – ‘Correction of the configuration’ with silver triflate and MeOH

Due to the anomeric mixture formation with PCl_3 , imidazole and Et_3N and a moderate yield in the 2-step synthesis (formation of the H-phosphonate and ‘correction of the configuration’) another method was tried for the formation of the H-phosphonate **34**.

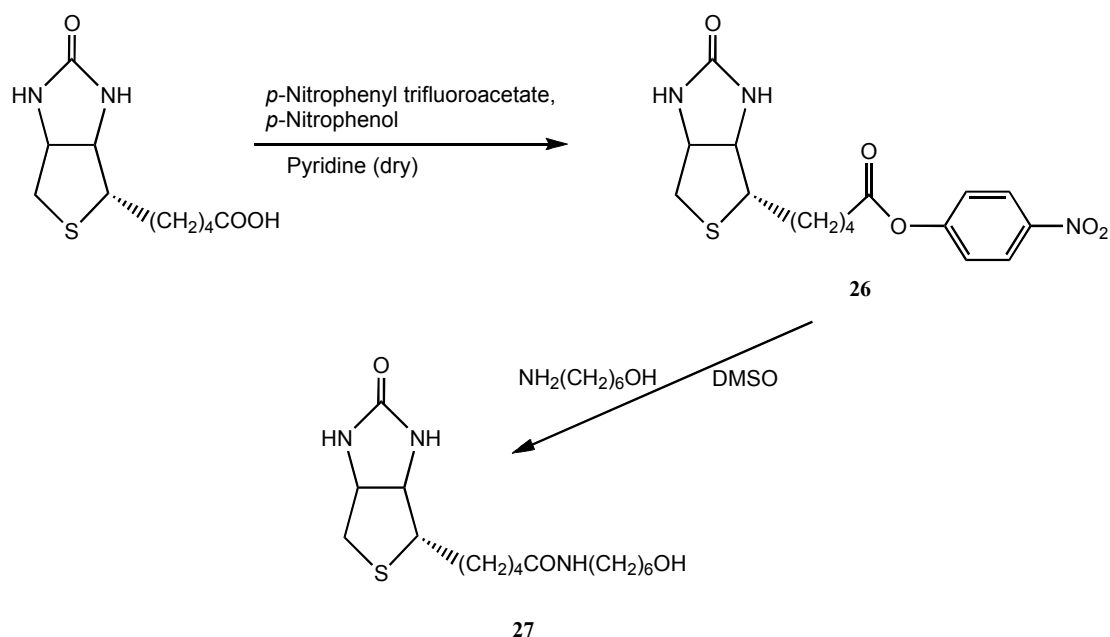


Scheme 16 – Preparation of the disaccharide H-phosphonate with salicyl chlorophosphate

This method ^{[103][104]} used salicyl chlorophosphite and Et₃N in acetonitrile reacting with derivative **33** (Scheme 16) for 1 h at room temperature, followed by hydrolysis with aq. TEAB buffer solution (pH 7.8). After purification the H-phosphonate **34** was isolated in a 98% yield and by ³¹P and ¹H NMR it could be seen that there was only the α -anomer. As this method gave pure α -H-phosphonate, there was no need to correct the configuration.

This 2,3,6-tri-*O*-benzoyl-4-*O*-[2,3,4-tri-*O*-benzoyl-6-*O*-(*p,p'*-dimethoxytrityl)- β -D-galactopyranosyl]- α -D-mannopyranosyl hydrogenphosphonate **34** is the first building block and also the repeat unit in the synthetic strategy. The next building block to synthesise is the biotin component.

2.3.6 Synthesis of 6-*N*-Biotinylaminohexanol **27**



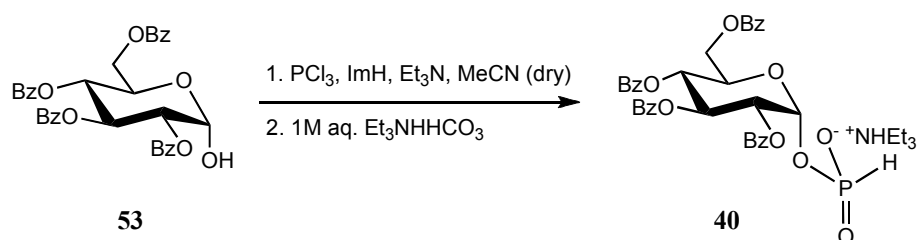
Scheme 17 – Preparation of 6-*N*-biotinylaminohexanol **27**

Synthesis of the biotin derivative **27** (Scheme 17) was achieved by converting D-biotin to the *p*-nitrophenyl ester **26** and then addition of the spacer molecule 6-aminohexanol^[106]. Compound **27** is the building block that will react with the disaccharide H-phosphonate to form the first of the target molecules. A mixture of D-biotin, *p*-nitrophenyl trifluoroacetate and *p*-nitrophenol in pyridine was heated to 55 °C for 2 h^{[105][106]}. The D-biotin *p*-nitrophenyl ester **26** was recrystallised from ethanol, giving a yield of 72 % of the crystals. The ester **26** was added to 6-aminohexanol in DMSO, and the mixture was left for 20 h^[105] at room temperature. The derivative **27** was precipitated by the addition of acetone and diethyl ether giving **27** in a good 80 % yield. This gave our final building block

in this synthetic strategy leaving the next step the condensation between the protected disaccharide H-phosphonate **34** and the 6-*N*-biotinylaminohexanol **27**.

2.3.7 Synthesis of 6-*N*-Biotinylaminohexyl 2,3,6-tri-*O*-benzoyl-4-*O*-[2,3,4-tri-*O*-benzoyl-6-*O*-(*p,p'*-dimethoxytrityl)- β -D-galactopyranosyl]- α -D-mannopyranosyl phosphate, triethylammonium salt **42** (protected form of the target compound **1**)

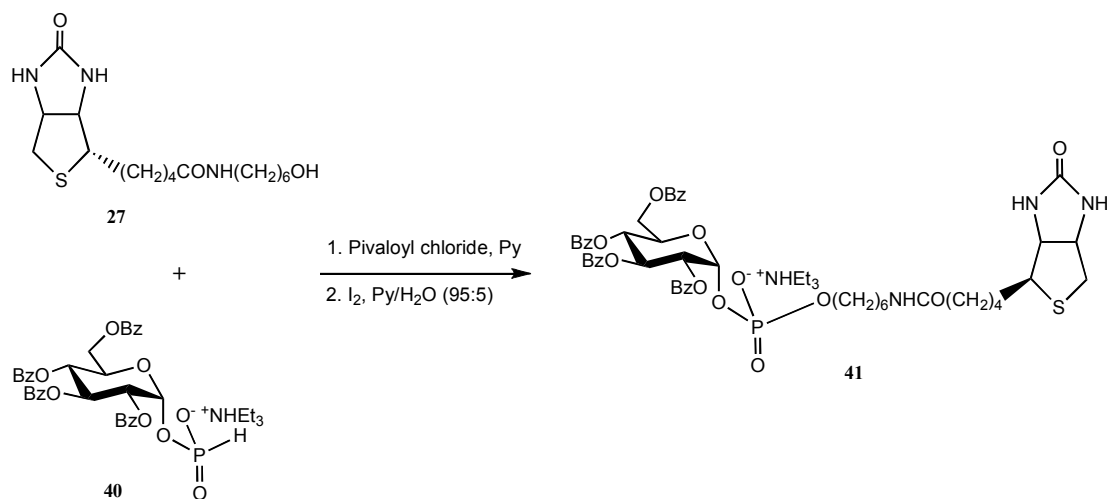
Before using the valuable disaccharide H-phosphonate **34** for the condensation with the biotin derivative **27**, a trial experiment using 2,3,4,6-tetra-*O*-benzoyl- α -D-glucopyranosyl H-phosphonate **40** (Scheme 19) was performed. Compound **40** (Scheme 18), in turn, was synthesised from the *O*-benzoylated D-glucose hemiacetal **53**, which was kindly donated by Dr Amina Fersia.



Scheme 18 – Preparation of the protected glucosyl H-phosphonate **40**

This was converted to the H-phosphonate derivative **40**^{[107][108]} by reacting it with triimidazolylphosphine formed *in situ* from PCl₃ and imidazole in the presence of

triethylamine, followed by hydrolysis with aq. TEAB buffer solution (pH 7.8). This gave the monosaccharide H-phosphonate in a good 78 % yield. The next step was to condense this compound with the biotinyl derivative **27** (Scheme 19).

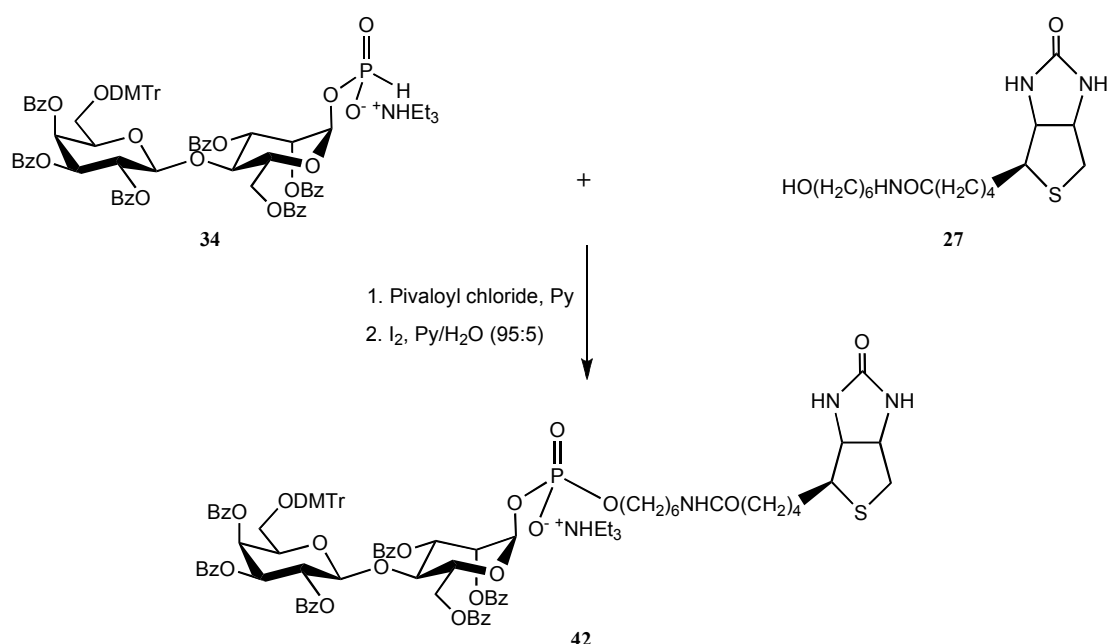


Scheme 19 – Preparation of 6-*N*-biotinylaminohexyl 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl phosphate

41

The compounds **27** and **40** were combined together using pivaloyl chloride as the condensing agent. After 90 min at room temperature, I₂ in a pyridine-water (95:5) solution was added to oxidise the intermediate H-phosphonic diester to the phosphate. After workup and purification on silica gel, the phosphodiester compound **41** was isolated in a 77 % yield and the structure was proved by ¹H, ¹³C and ³¹P NMR data. This trial experiment was a good indicator that the condensation of the larger disaccharide unit should progress well and the conditions are well suited.

The condensation of the protected disaccharide H-phosphonate **34** and the modified biotin **27** (Scheme 20) was carried out in the same conditions as the model synthesis (Scheme 19) had been run. It was noticed after the oxidation step that the biotin containing sugar derivative **42** was very polar on TLC and running just slightly above the unreacted biotin compound **27**. This made the purification of the biotin sugar phosphate **42** a lot more complicated to get a pure compound. On TLC it was also seen there was much cleavage of the H-phosphonate group in compound **34**, which resulted in the disaccharide hemiacetal **33** as the major product. After workup and purification on silica gel a small amount of target compound **42** was recovered (8 %). This was just enough to run ^1H and ^{31}P NMR spectra to prove the structure of the product.

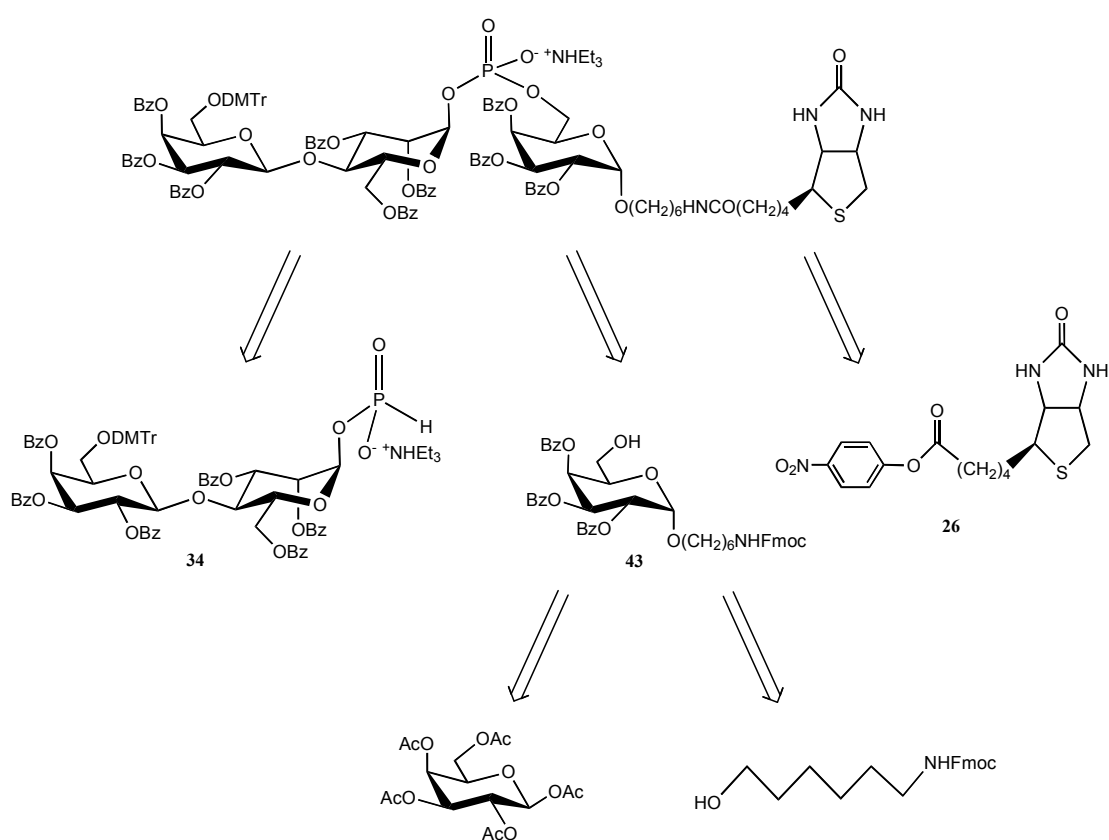


Scheme 20 – Preparation of 6-*N*-biotinylaminoheptyl 2,3,6-tri-*O*-benzoyl-4-*O*-[2,3,4-tri-*O*-benzoyl-6-*O*-(*p,p'*-dimethoxytrityl)]-β-D-galactopyranosyl]-α-D-mannopyranosyl phosphate

Due to the very low yield of the compound and the difficulty in purifying biotinylated protected carbohydrate phosphates it was decided to abandon the method of biotin integration through a phosphate group at the beginning of the chemical chain elongation. Instead it was decided to look for a method introducing biotin later in the synthetic scheme thus avoiding the polarity issues that led to purification problems.

2.4 Attempted synthesis of biotinylated phosphosaccharide compounds using a monosaccharide bridge to biotin

A retrosynthetic scheme that incorporated the biotin moiety later in the synthetic scheme, while reducing the polarity of the overall protected molecule, was identified (Scheme 21).

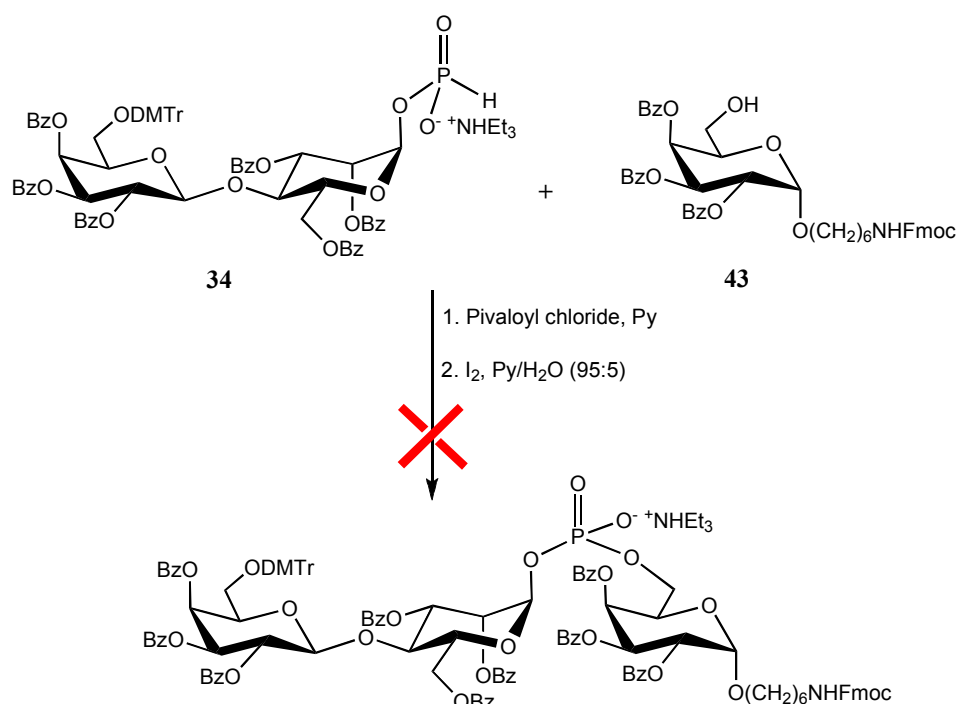


Scheme 21 – Retrosynthetic approach using the monosaccharide bridge

The synthetic strategy enabled integration of the biotin moiety as a final step, allowing a stepwise or blockwise building approach to be used for chain elongation by simple removal of the *p,p'*-dimethoxytrityl protecting group. The D-galactose linker derivative **43** can be coupled with the repeat unit H-phosphonate **34** by a condensation at the position 6. Once the corresponding trisaccharide monophosphate is formed, removal of the Fmoc protection from amino group in the linker and coupling with the biotin *p*-nitrophenyl ester **26** should be possible. The first reaction to carry out was to test the coupling between the disaccharide H-phosphonate **34** and the Gal-linker-Fmoc derivative **43**.

2.4.1 Attempted condensation of 2,3,6-tri-O-benzoyl-4-O-[2,3,4-tri-O-benzoyl-6-O-(*p,p'*-dimethoxytrityl)- β -D-galactopyranosyl]- α -D-mannopyranosyl H-phosphonate **34 and Gal-linker-Fmoc derivative **43****

The reaction was to form an inter-saccharide phosphate compound, which had been reported many times ^[67], but had never been attempted in the presence of the *N*-Fmoc protecting group. Compound **43** was synthesised and kindly donated by Dr Dimity Yashunsky for use in this experiment. Compounds **34** and **43** were combined (Scheme 22) and the reaction was run under standard condensation and oxidation conditions: pivaloyl chloride as the condensing agent and iodine in pyridine-water (95:5) for oxidation.

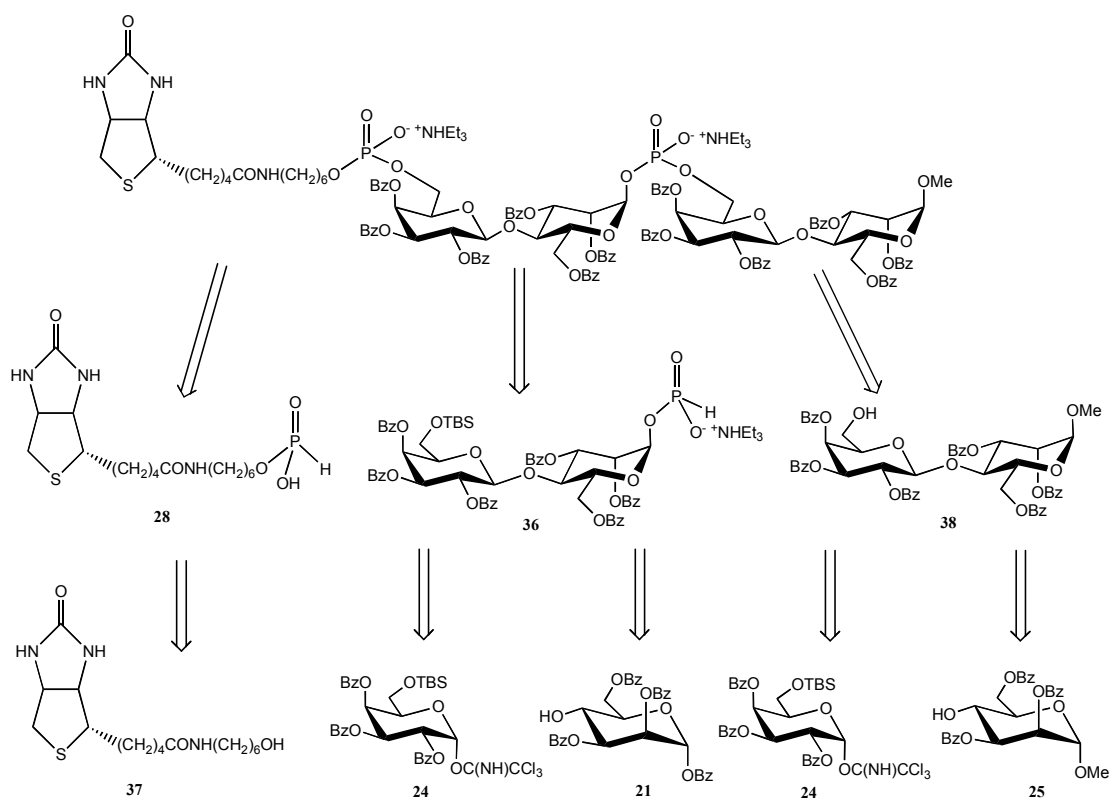


Scheme 22 – Attempted coupling of the disaccharide H-phosphonate and Gal-linker-Fmoc derivative

It could be seen on TLC plates that there was no reaction between the H-phosphate and the free hydroxyl of the galactose derivative. There have been many reactions, which have showed forming a inter-sugar phosphate link being successful ^[67], in this case it is unknown what stopped the reaction. One idea is that the *N*-Fmoc protection interfered with the condensation somehow, but due to the lack of previous experiments of this type containing this protecting group it remains just an idea. Due to this critical step not working, it was decided to abandon this synthetic strategy, and design another that would allow us to introduce the biotin moiety at the final step of the chemical chain elongation.

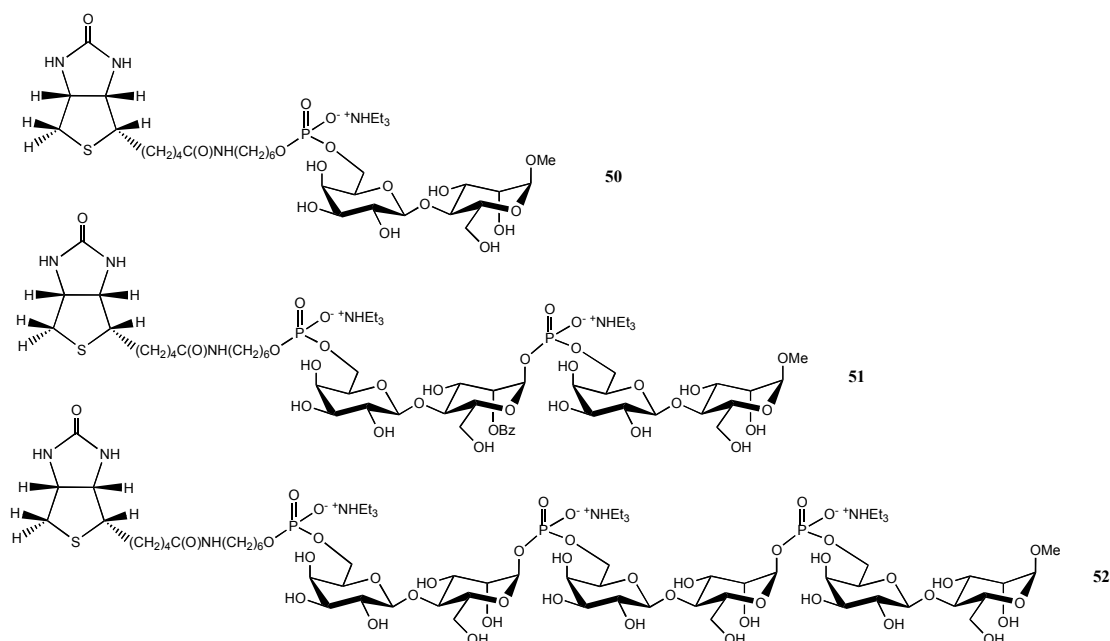
2.5 Synthesis of the target compounds 50, 51 and 52 with biotin moiety at the non-reducing end of the chain

A novel approach was needed to circumvent the issues biotin was causing during the synthesis. It was accomplished by (i) changing the way the biotin moiety was introduced, from the reducing end (Scheme 8) to the non-reducing end of the chain and (ii) using the H-phosphonate biotin derivative **28** (Scheme 23).



Scheme 23 – Retrosynthetic approach introducing biotin to the non-reducing end of the chain

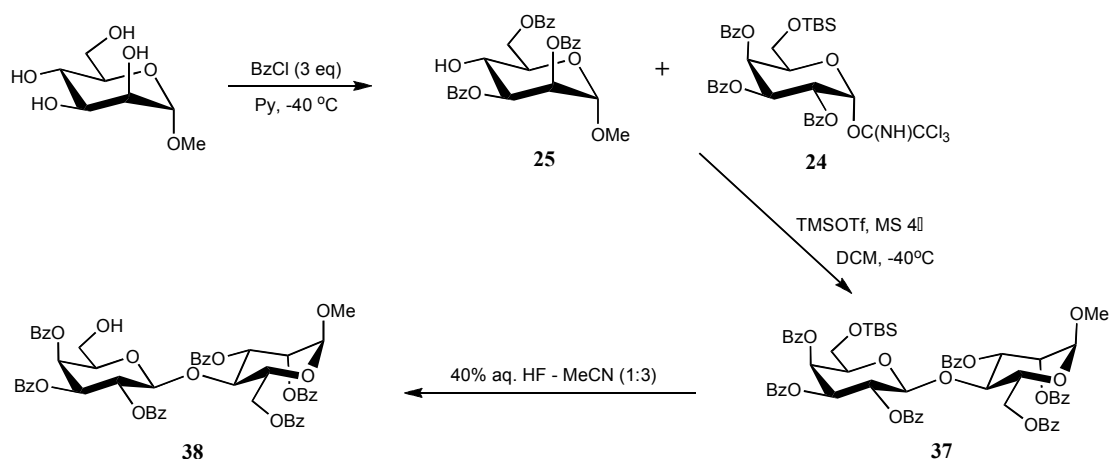
This would allow stepwise chain elongation with the disaccharide H-phosphonate **36** (as well as the disaccharide H-phosphonate **34**) to form the larger phosphosaccharide units and biotin integration at the final step before the total deprotection. It was shown in Vishwakarma et al.^{[86][87]} that the silyl protecting group at D-galactose position 6 of Gal-Man-phosphate protected derivatives could be easily removed in presence of the phosphate group. So, it was decided to keep the silyl in place in compound **30** (Scheme 12) rather than remove it and replace with a DMT protection as in former schemes. The disaccharide **30** was then converted to the disaccharide H-phosphonate **36** (see below). By adding biotin at the non-reducing end we require a permanent blocking group at the anomeric position of the terminal D-mannose unit, which could be a methyl glycoside. Thus, a methyl bioside derivative **38** should be prepared and used as the starting monohydroxyl disaccharide derivative for elongation of the phosphoglycan chain. By changing to this synthetic strategy this modifies our target molecules (Scheme 24), which now seem to be compounds **50**, **51** and **52** containing one, two and three repeats, respectively, and all containing the biotin moiety linked via a 6-aminohexyl linker to a phosphate at the non-reducing end of the chain.



Scheme 24 – Modified target compounds **50**, **51** and **52** containing biotin moiety at the non-reducing end of the chain

2.5.1 Synthesis of methyl 2,3,6-tri-*O*-benzoyl-4-*O*-(2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl)- α -D-mannopyranoside **38**

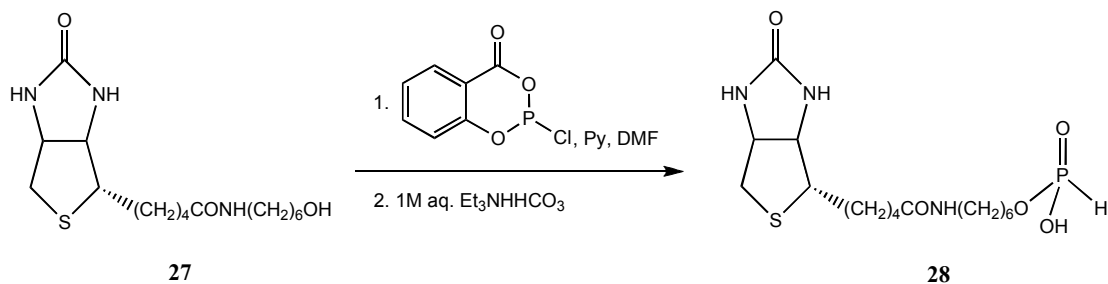
Formation of the methyl bioside derivative **38** (Scheme 25) was performed using the glycosyl donor **24** from the previous glycosylation to form the repeat unit derivatives. This was coupled with the methyl mannoside derivative **25** ^{[110][111]} to provide the protected disaccharide **37**. The next step was to remove the silyl protecting group at D-galactose position 6 to provide the hydroxyl for further phosphorylation.



Scheme 25 – Preparation of methyl glycoside derivatives **25**, **37** and **38**

Methyl α -D-mannopyranoside was reacted with a strict 3 eq. of benzoyl chloride in pyridine at -40°C . After 21 h the reaction was quenched, worked-up and purified to give the tribenzoate **25** in a good 71% yield. The glycosyl acceptor **25** was combined with the glycosyl donor **24** and TMSOTf to catalyse the glycosylation. It was found by doing repeat experiments the optimum temperature for this reaction was -40°C . After purification disaccharide **37** was isolated in a 68 % yield. Treatment with aq. HF in acetonitrile for 1 h was enough to remove the silyl protection at the 6 position of the D-galactose in a quantitative yield. This gave the required monohydroxyl disaccharide compound **38** for further condensation with an H-phosphonate derivative.

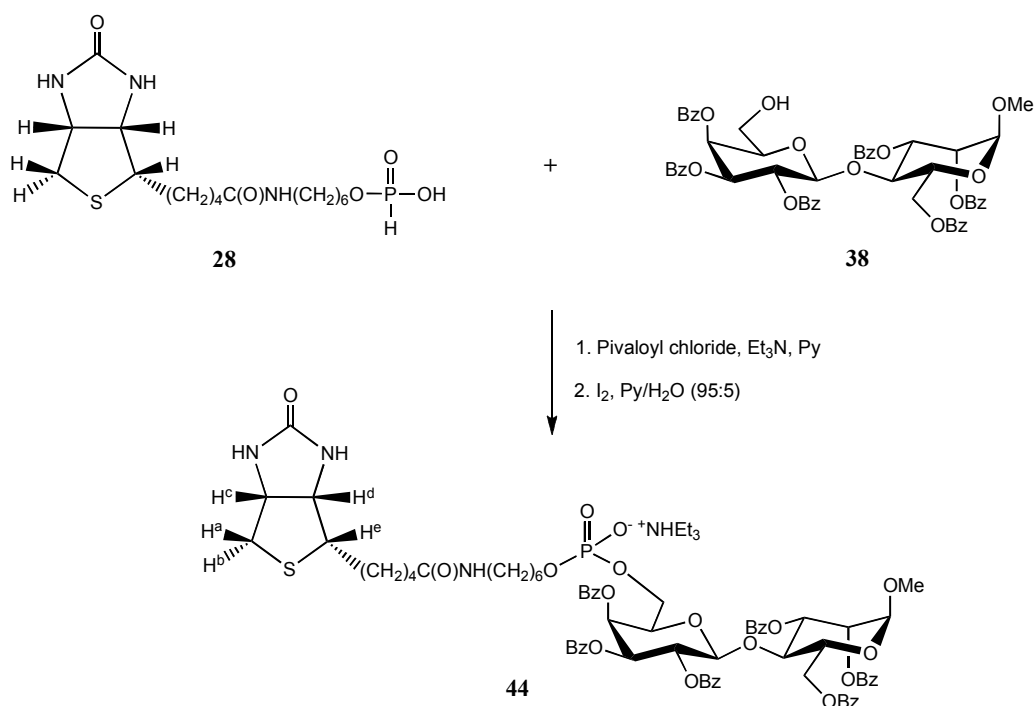
2.5.2 Synthesis of 6-*N*-Biotinylaminohexyl hydrogenphosphonate **28**



Scheme 26 – Preparation of the biotin H-phosphonate derivative **28**

Formation of the biotinylaminohexyl H-phosphonate **28** (Scheme 26) was achieved by reacting biotin-linker derivative **27** with salicyl chlorophosphate in DMF ^[106]. After 10 min the reaction was quenched with 1M TEAB buffer solution and diethyl ether was added to the reaction mixture, and it was then left in the fridge for 16 h. This allowed the biotin derivative to precipitate out of solution giving compound **28** as an acid (H⁺-form) in a 90 % yield.

2.5.3 Synthesis of methyl 2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranoside 6-(6-*N*-biotinylaminohexyl phosphate), triethylammonium salt **44**



Scheme 27 – Formation of methyl 2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranoside 6-(6-*N*-biotinylaminohexyl phosphate) **44**

Before the start of the condensation reaction both compounds **38** and **28** (Scheme 27) were combined and dried by evaporation of pyridine therefrom to ensure strictly anhydrous conditions. The reaction was started by dissolving the mixture in pyridine, and triethylamine was added to convert the H-phosphonate **28** to the corresponding triethylammonium salt. Pivaloyl chloride was added to the mixture as the condensing agent, and after 60 min it was seen by TLC that compound **38** had been consumed.

Therefore the reaction product, the corresponding H-phosphonic diester, was oxidised by the addition of iodine in pyridine-water. After work-up and purification on silica gel, the protected biotin linked disaccharide phosphate **44** was isolated in an 84 % yield. The compound's structure was proven by analysis of the ^1H , ^{13}C and ^{31}P NMR (see Fig 21) and high resolution mass spectrometry data. This gave the protected form of the first of the target compounds, the one repeat unit linked to biotin in the *O*-benzoylated form. Deprotection of the compound was done once the other protected target compounds had been synthesised. With the successful synthesis of the first protected target compound, the next step was to form the two repeat unit compound.

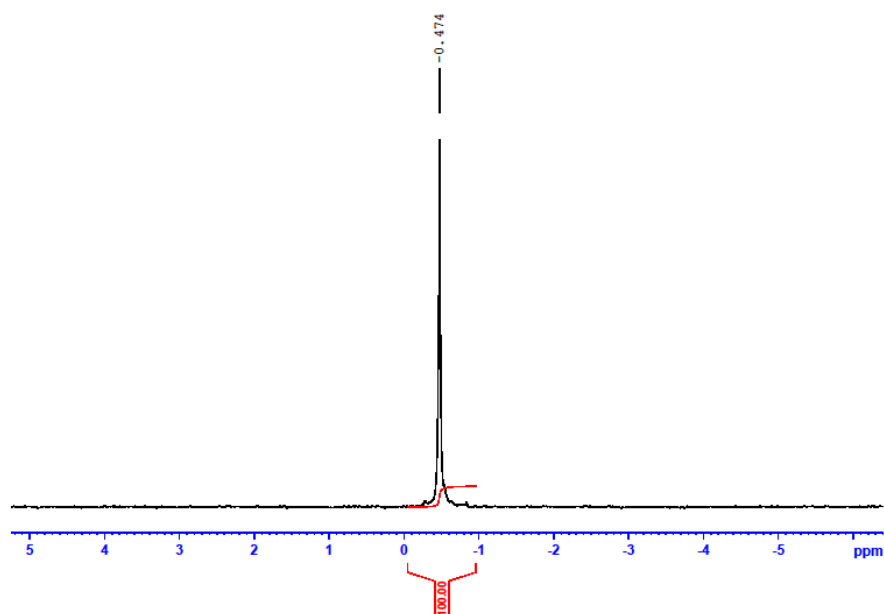
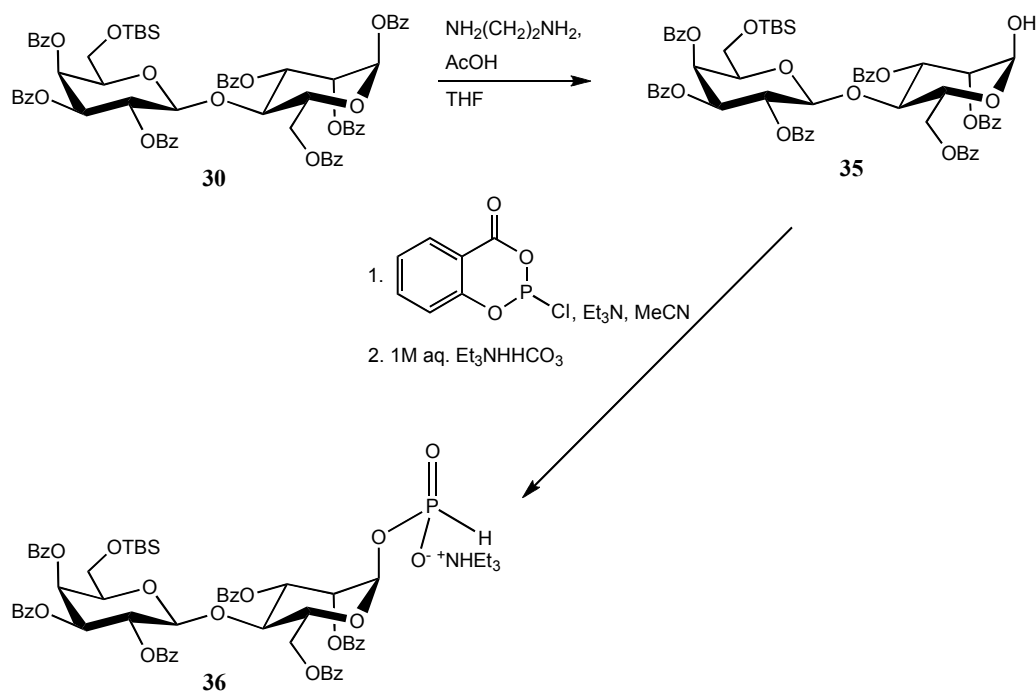


Fig 21: ^{31}P NMR spectrum of **44**

2.5.4 Synthesis of 2,3,6-Tri-*O*-benzoyl-4-*O*-(2,3,4-tri-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl- β -D-galactopyranosyl)- α -D-mannopyranosyl hydrogenphosphonate, triethylammonium salt **36**

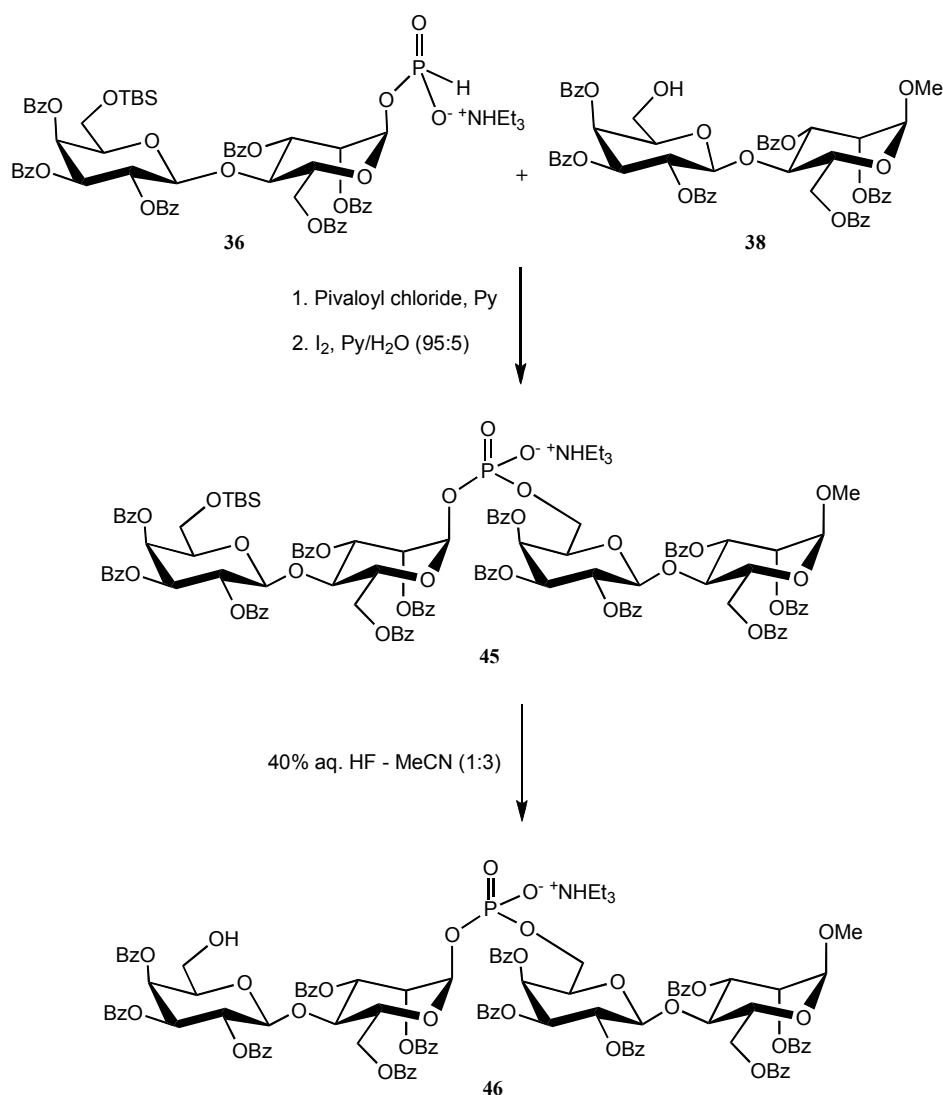


Scheme 28 – Preparation of TBS protected disaccharide H-phosphonate **36**

Derivative **30** (for preparation, see scheme 12) was converted to the hemiacetal **35** by the reaction with ethylenediamine and acetic acid in THF (Scheme 28). After the work-up and purification this gave the product in an 87% yield. Hemiacetal **35** was then used for the formation of H-phosphonate derivative **36** by reacting it with salicyl chlorophosphate in acetonitrile. When it was quenched with TEAB buffer solution, worked-up and purified, this gave the desired product **36** in a 98 % yield. This H-phosphonate compound was used

as the preferred repeat unit derivative for the next step of the synthesis due to lower number of steps in the synthesis compared with its DMT containing counterpart.

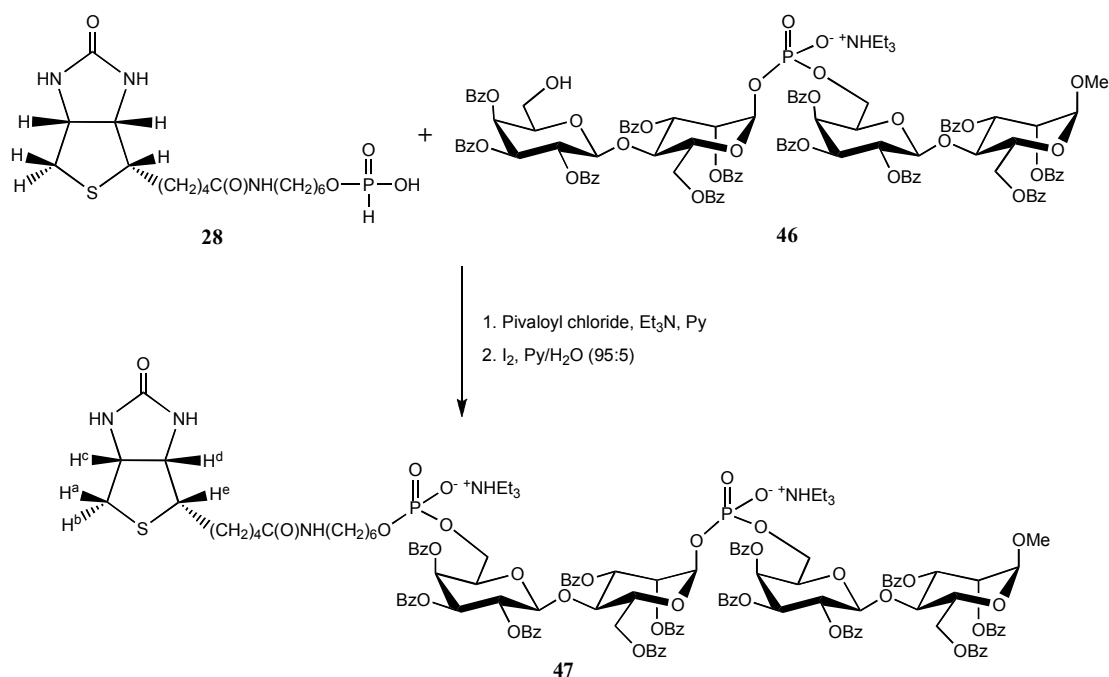
2.5.5 Synthesis of methyl 2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranoside 6-[2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranosyl phosphate] triethylammonium salt **46**



Scheme 29 – Formation and deprotection of the tetrasaccharide monophasate **45**

The condensation of compounds **36** and **38** (Scheme 29) was carried out under standard conditions^[112], co-evaporations with pyridine to ensure dryness of the compounds, the use of pivaloyl chloride as the condensing agent and iodine in pyridine-water (95:5) for the oxidation. After purification on silica gel this gave the protected tetrasaccharide phosphate **45** in a very good 82 % yield. To remove the silyl protecting group from the derivative **45**, treatment with a mixture of 40 % aq. HF and acetonitrile (1:3) at 0 °C was carried out for 2 h. This removed the silyl group while not harming the phosphate linkage, giving the tetrasaccharide phosphate **46** in a 90 % yield.

2.5.6 Synthesis of methyl 2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranoside 6-[2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranosyl phosphate 6-(6-*N*-biotinylaminoethyl phosphate)], bistriethylammonium salt **47**



Scheme 30 – Preparation of the protected biotinylated phosphosaccharide **47**

The condensation of the derivatives **28** and **46** (Scheme 30) was performed under standard condensation conditions, but with the addition of triethylamine during the condensation step. This was due to the fact that the biotin H-phosphonate derivative **28** is an acid and not the triethylammonium salt form. The reaction ran for 1 h and the product was then oxidised for 40 min. After the work-up and purification the protected biotin linked tetrasaccharide diphosphate **47** was isolated in a 79 % yield. The structure was confirmed through the ¹H, ¹³C and ³¹P NMR data as well as the high resolution ES-MS

data. It can be seen very clearly in the ^{31}P NMR spectrum (Fig 22) the two peaks at -4.39 and -0.64 ppm.

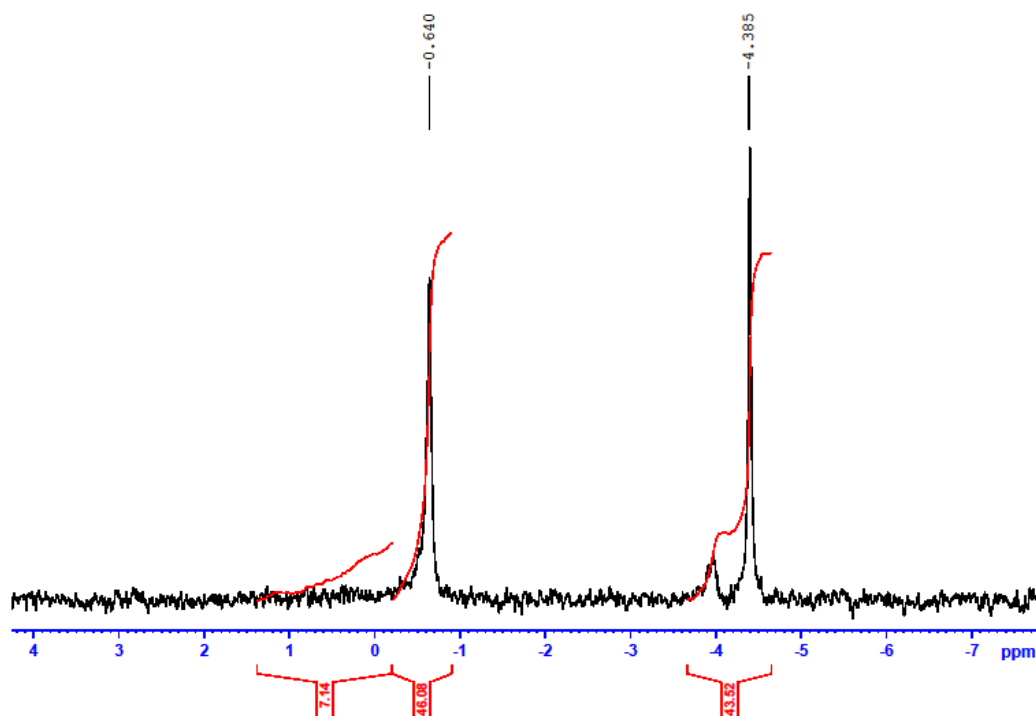
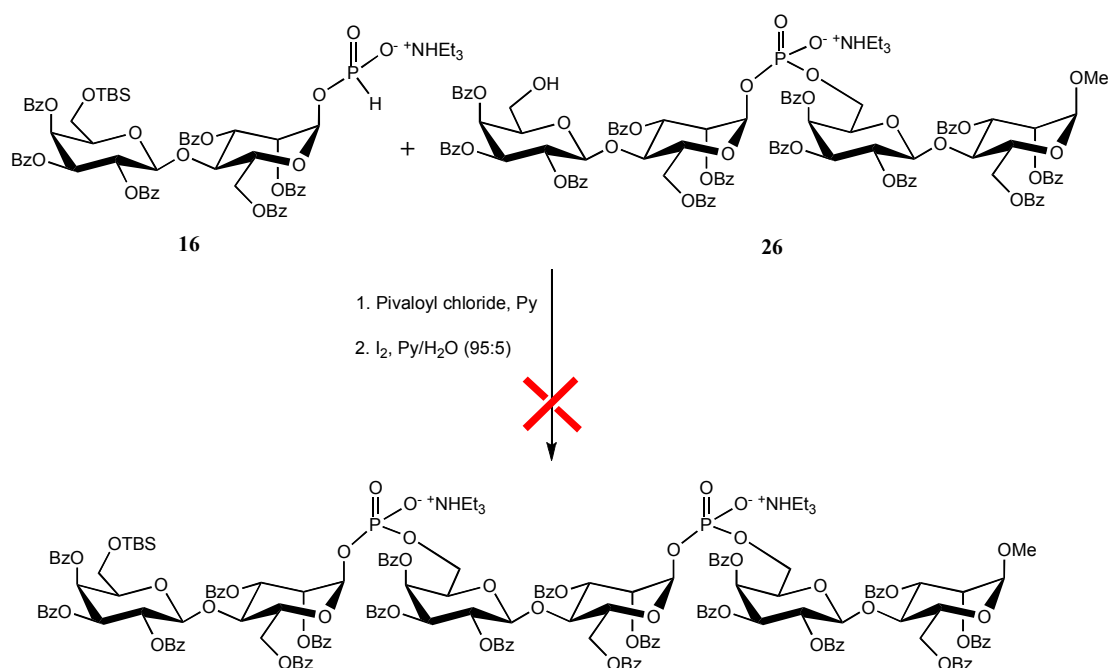


Fig 22 : ^{31}P NMR spectrum of compound **47**

The peak seen at -0.64 ppm is the phosphate bridge between biotin and the last sugar unit. The peak at -4.39 ppm is very typical for the inter-saccharide phosphate signal, which is expected to be seen for the phosphate bridge between the disaccharide units. High resolution mass spec gave two pseudo-molecular ions for the protected biotin linked tetrasaccharide diphosphate **47**, the first being at m/z 1206.2823 $[\text{M} - 2\text{Et}_3\text{N} - 2\text{H}]^{2-}$ and the other at m/z 2412.5840 $[\text{M} - 2\text{Et}_3\text{N} - \text{H}]^-$.

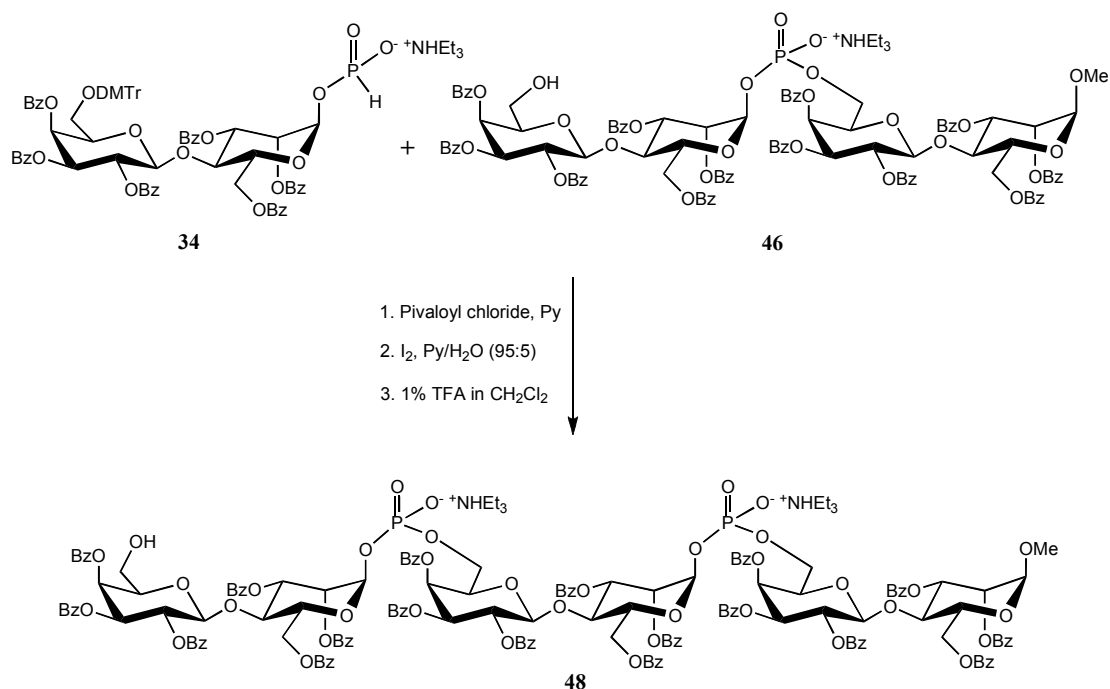
2.5.7 Synthesis of methyl 2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranoside 6-{2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranosyl phosphate 6-[2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranosyl phosphate]}, bistriethylammonium salt **48**



Scheme 31 – Attempted coupling of the disaccharide H-phosphonate **36** and the tetrasaccharide monophosphate **46**

The formation of the protected hexasaccharide diphosphate derivative using the TBS protected disaccharide H-phosphonate **36** and the tetrasaccharide monophosphate **46** (Scheme 31) was attempted many times under the normal condensation and oxidation conditions. The amount of the pivaloyl chloride and iodine were varied to try and help the reaction, but this had no success. The condensation with the TBS containing derivative **36**

did not work, so it was decided to attempt a coupling using the DMT protected disaccharide H-phosphonate **34** (Scheme 32). The disaccharide H-phosphonate **34** was synthesised using the alternative strategy discussed in the Section 2.3.4.2.



Scheme 32 – Preparation of the hexasaccharide diphosphate **48** using the DMT protected disaccharide H-phosphonate **34**

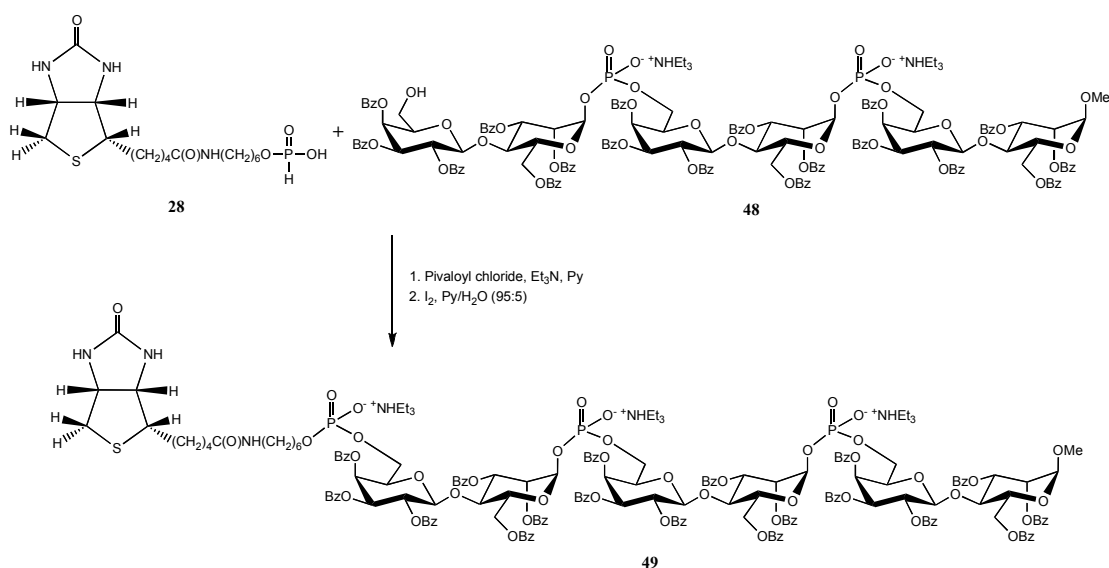
Using the DMT containing H-phosphonate **34** and the tetrasaccharide phosphate **46** the condensation was attempted under normal condensation and oxidation conditions. After 2 h of condensation and 3 h of oxidation it could be seen by TLC that a new DMT product had been formed (DMT is very noticeable due to the orange colour given after acid visualisation of the TLC plate). After the work-up, the residue was dried and treated with 1 % trifluoroacetic acid in DCM for 2 min at 0 °C to cleave the acid sensitive DMT

protecting group. The reaction mixture was worked-up and the residue was purified giving the hexasaccharide diphosphate **48** in a respectable 62 % yield. The structure was proved by ^1H , ^{13}C and ^{31}P NMR data. In the ^{31}P NMR spectrum it was possible to see both inter-saccharide phosphate signals at -4.47 and -4.38 ppm. High resolution mass spectrometry analysis also showed two pseudo-molecular ions at m/z 1517.8813 [$\text{M} - 2\text{Et}_3\text{N} - 2\text{H}$] $^{2-}$ and 3035.8390 [$\text{M} - 2\text{Et}_3\text{N} - \text{H}$] $^-$.

The reason for the DMT derivative **34** working and the TBS derivative **36** not working in the condensation reaction with the tetrasaccharide phosphate **46** remains unknown. It could be to do with the size of the protecting groups and the effect this may have on the rest of the molecule. The DMT group is much larger compared with the TBS group and could influence the rest of the molecule somehow.

2.5.8 Synthesis of methyl 2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranoside 6-{2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranosyl phosphate 6-[2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranosyl phosphate 6-(6-*N*-biotinylaminohexyl phosphate)]}, triethyammonium salt **49**

The coupling of the biotin H-phosphonate **28** and the hexasaccharide diphosphate **48** was carried out in the normal condensation and oxidation conditions, with the addition of the triethylamine before (to convert compound **28** to the triethyammonium salt) before the pivaloyl chloride was added. After 2 h for the condensation step and 3 h for the oxidation step, it could be seen by TLC indicated the appearance of a strong new spot.



Scheme 33 – Preparation of the protected biotinylated phosphosaccharide **49**

Work-up and purification gave the desired protected biotin linked hexasaccharide triphosphate **49** in a good yield of 64 %. The structure was proved by ^1H , ^{13}C and ^{31}P NMR data. In the ^{13}C NMR spectrum it was possible to see resonance signals for all the anomeric carbon atoms: 93.94 and 94.04 ppm (2 x br, C-1 Man' & C-1 Man''), 98.35 ppm (C-1 Man), 101.03 ppm (C-1 Gal'), 101.22 and 101.27 ppm (C-1 Gal & C-1 Gal''). In the ^{31}P NMR spectrum (Fig 23) three signals were seen at -4.66, -4.42 and -0.59 ppm, with the biotin-sugar phosphate linkage at -0.59 and the inter-saccharide phosphate linkages at -4.66 and -4.42. This is the final of the targeted protected compounds to be successfully synthesised.

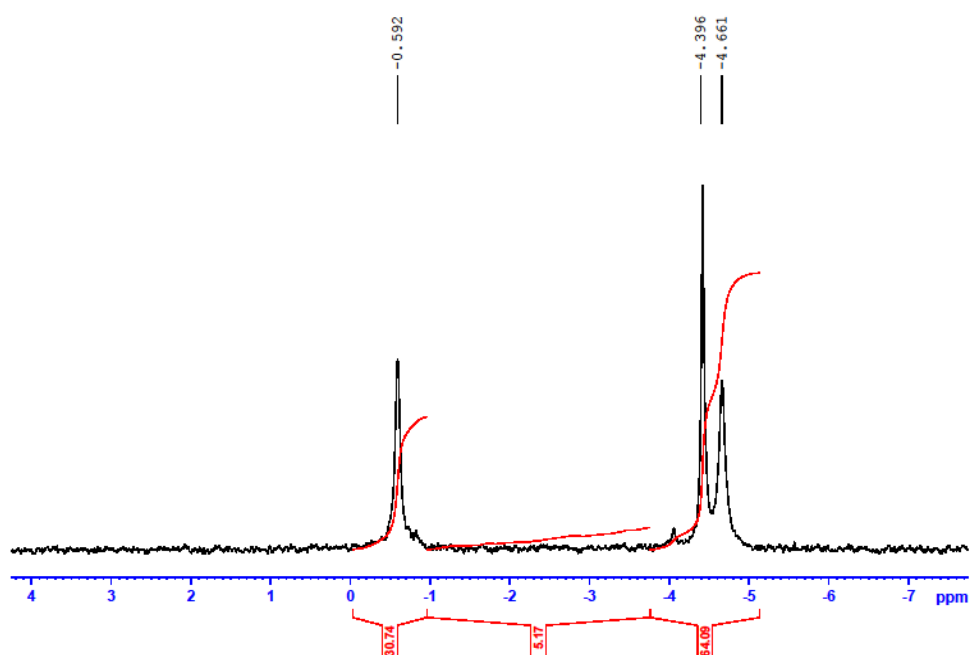
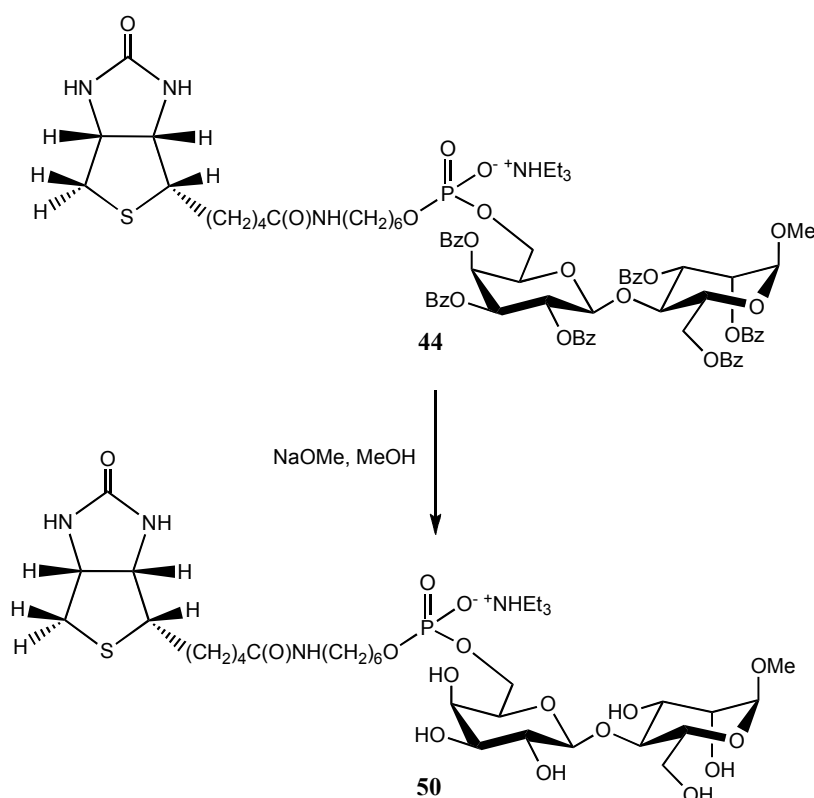


Fig 23: ^{31}P NMR spectrum of compound **49**

2.5.9 Synthesis of methyl β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranoside 6-(6-*N*-biotinylaminoethyl phosphate), triethylammonium salt **50** (the first target compound)

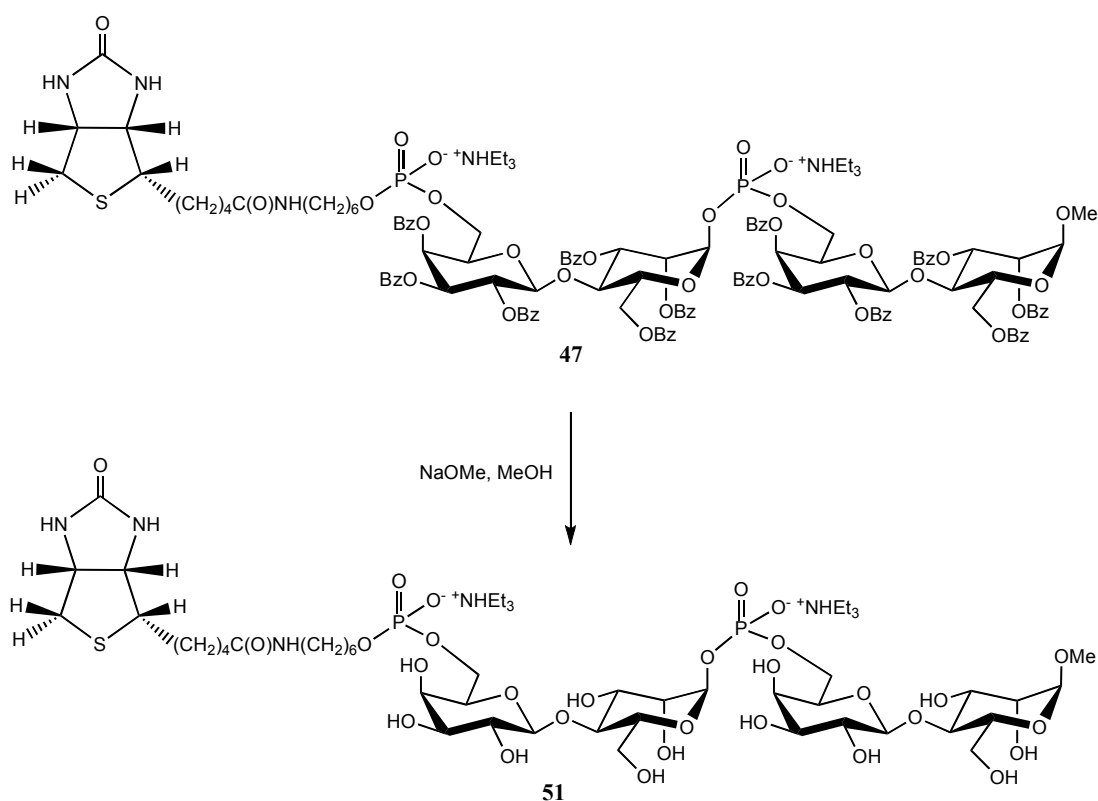
The full deprotection of compound **44** using sodium methoxide in methanol (to remove all the benzoate protecting groups) yielded the fully deprotected methyl β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranoside 6-(6-*N*-biotinylaminoethyl phosphate), triethylammonium salt **50** (95 %), i.e. the first target compound containing one repeat unit linked to biotin moiety (Scheme 27).



Scheme 34 – Preparation of the target compound **50**

The structure was verified by ^1H , ^{13}C and ^{31}P NMR. High resolution mass spec gave m/z 760.2733 $[\text{M} - \text{Et}_3\text{N} - \text{H}]^-$ which confirmed the mass of the deprotected compound.

2.5.10 Synthesis of methyl β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranoside 6-[β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranosyl phosphate 6-(6-*N*-biotinylaminohexyl phosphate)], bistriethylammonium salt **51 (the second target compound)**



Scheme 35 – Preparation of the target compound **51**

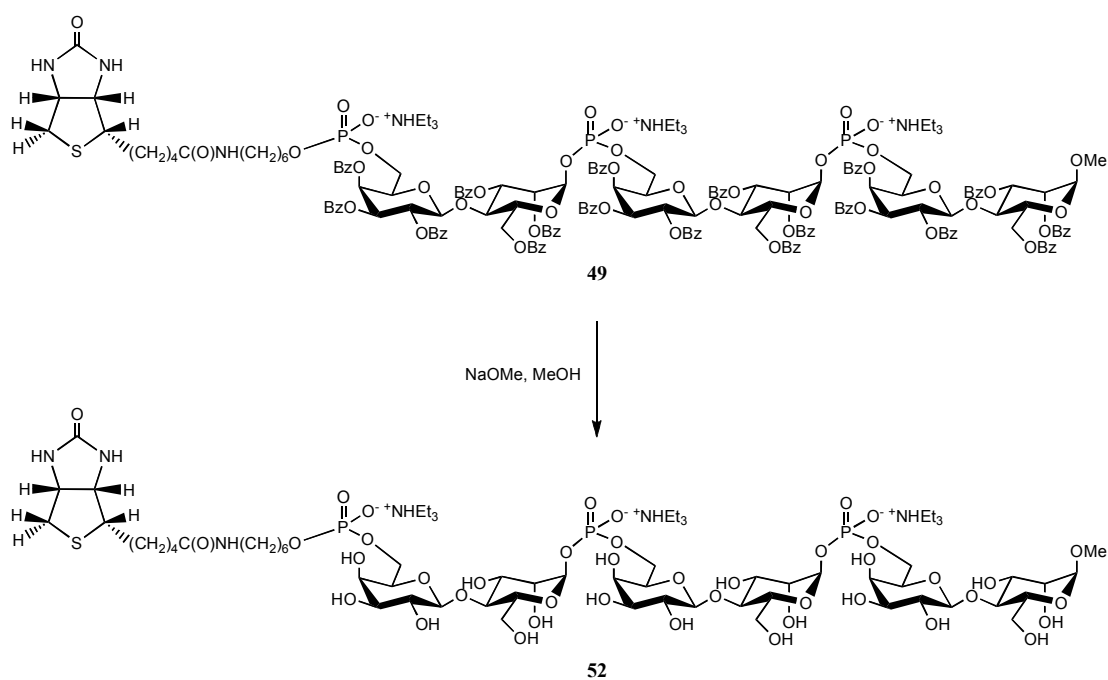
The full deprotection of compound **47** using sodium methoxide in methanol (to remove all the benzoate protecting groups) yielded the fully deprotected methyl β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranoside 6-[β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-

mannopyranosyl phosphate 6-(6-*N*-biotinylaminohexyl phosphate)],
bistriethylammonium salt **51** (98 %), i.e. the second target compound containing two repeat units linked to biotin moiety (Scheme 35). The structure was verified by ^1H , ^{13}C and ^{31}P NMR. High resolution mass spec gave m/z 1164.3488 $[\text{M} - 2\text{Et}_3\text{N} - \text{H}]^-$ which confirmed the mass of the deprotected compound.

2.5.11 Synthesis of methyl β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranoside 6-{ β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranosyl phosphate 6-[β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranosyl phosphate

6-(6-*N*-biotinylaminohexyl phosphate)]}, tris(ethylammonium) salt **52 (the third target compound)**

The full deprotection of compound **49** using sodium methoxide in methanol (to remove all the benzoate protecting groups) yielded the fully methyl β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranoside 6-{ β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranosyl phosphate 6-[β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranosyl phosphate 6-(6-*N*-biotinylaminohexyl phosphate)]}, tris(ethylammonium) salt **52** (90 %), i.e. the third target compound containing three repeat units linked to the biotin moiety (Scheme 36). The structure was verified by ^1H , ^{13}C and ^{31}P NMR data. High resolution mass spec gave pseudo-molecular ions of m/z 1584.4125 $[\text{M} - 3\text{Et}_3\text{N} - \text{H} + \text{NH}_3]^+$ and 1669.9165 $[\text{M} - 2\text{Et}_3\text{N} - \text{H}]^+$ which confirmed the mass of the deprotected compound.



2.6.0 Biological Results

The deprotected target compounds (**50 – 52**) were then passed through to Prof. Mike Ferguson's lab in Dundee, where PhD student Lauren Sullivan carried out the experiments using the compounds. The first set of experimental data that was needed was to measure the antibody binding to the synthetic repeat structures; this was done by using an ELISA assay.

2.6.1 ELISA assay

The assay was setup using a 48-well plate that was coated in a neutra-avidin to enable binding of the biotin in the final compounds. All 3 target compounds were

run against each other and against biotin. Biotin was used as a control and to block the neutra-avidin on the plate from any side reactions with the antibodies. The antibody used were the monoclonal IVD3 that recognises the repeat unit structure were added first. The anti-mouse IgG horseradish peroxidise (HRP) which binds to the IVD3 antibody and when the HRP substrate is added and it gives out a chemiluminescence that is measured on an nVision spectrometer. An overview of the ELISA assay is shown below in Fig 27.

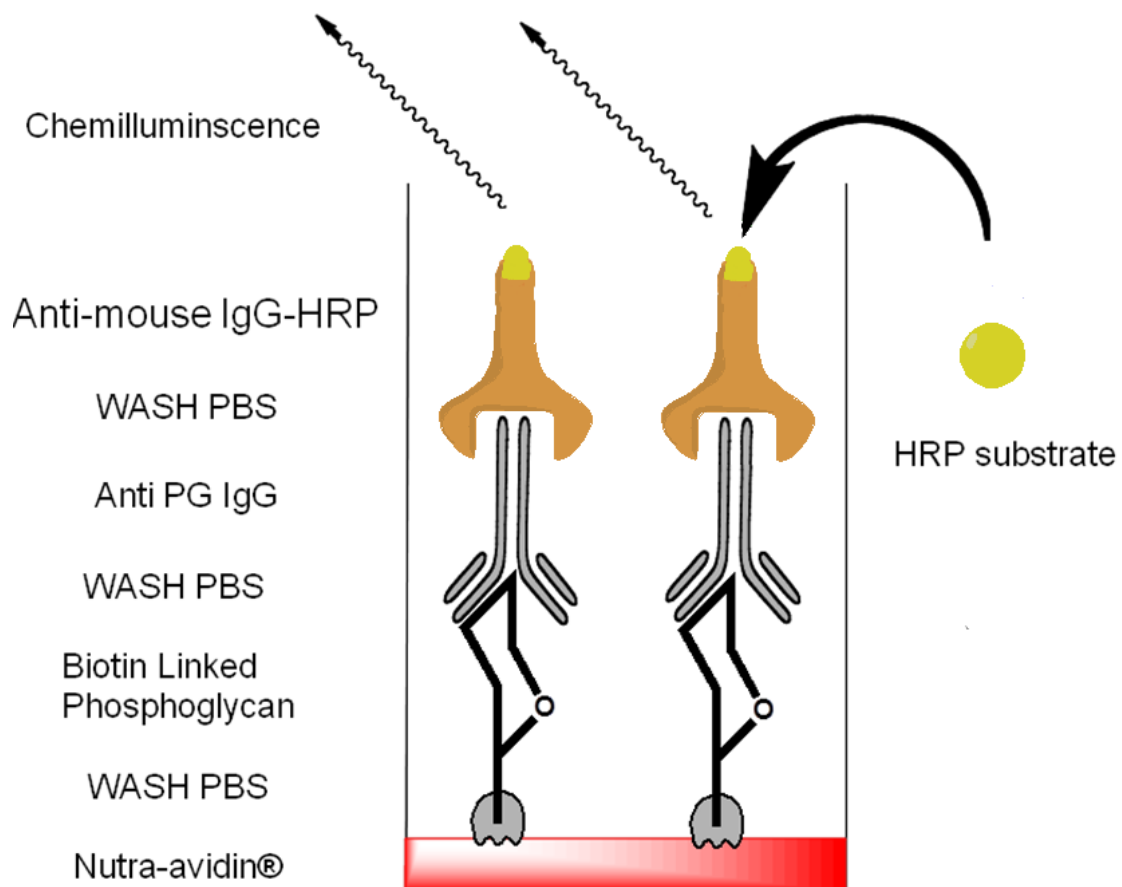


Fig 27 – The ELISA assay

2.6.2 ELISA results

The results from the ELISA assay can be seen in Figs 28 and 29. Two experiments were run in parallel, 1 was using an ascites sample and the other used purified IgG from the ascites. An ascites sample is the fluid from the peritoneal cavity of the animal in which the antibodies have been raised (in this case a mouse). The ascites contains a mixture of antibodies and many other things. This can be purified and the antibody need can be isolated, this is what was done to achieve pure IgG.

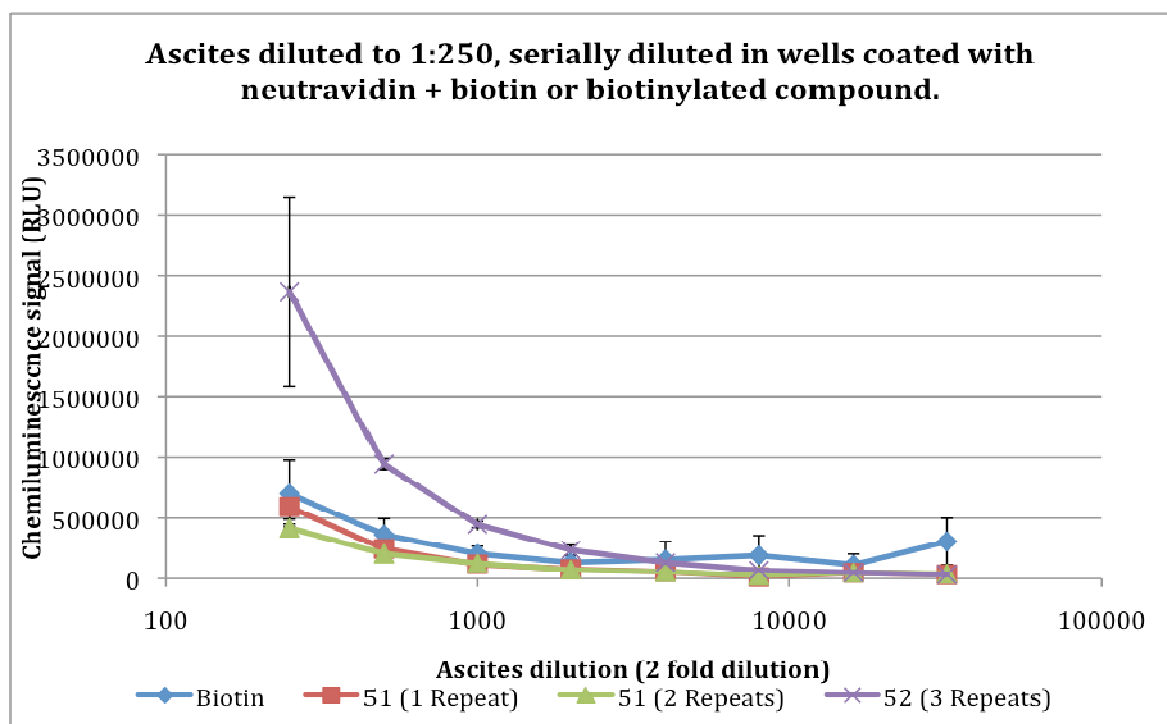


Fig 28 – ELISA results of ascites dilution

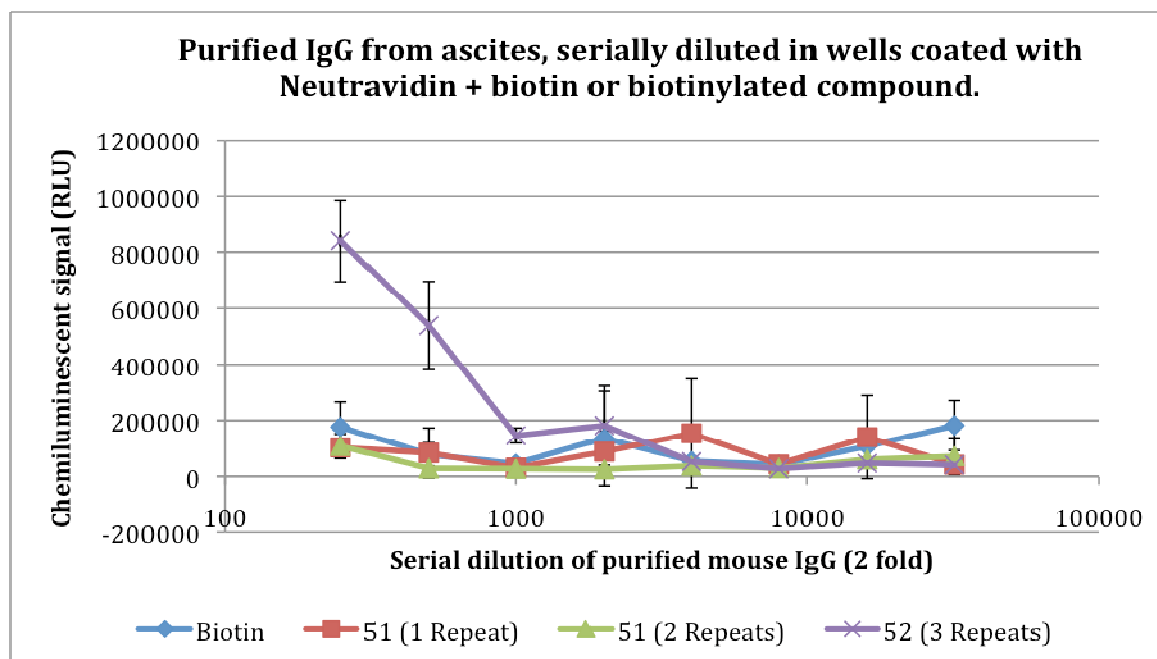


Fig 29 – ELISA results of purified IgG

It can be seen from the graphs that the compounds containing 1 or 2 repeat units did not perform well, with their signals similar to the pure biotin. The 3 repeat unit has a very good signal meaning there is antibody recognition and binding. The signal is much larger than the background or biotin only signal meaning there is very good interaction between the repeats and the antibody. This positive result from the compound containing 3 repeat units is very useful for the biochemical work and provides a foundation for further work. Now there is a synthetic phosphoglycan repeat unit with the biotin moiety attached providing the basis and possibility for further assay formations.

The further work that will be carried out involves the comparison of this binding, during an inhibition assay, by using the same assay as before but with the addition of phosphoglycan (PG) fragments to see if the anchored compounds or the PG

fragments bind the antibodies. This will mimic what will happen when urine containing PG fragments is used. The experiment will show if plate bound PG repeats can be inhibited by free PG repeats. This work is ongoing in the Lab of Mike Ferguson.

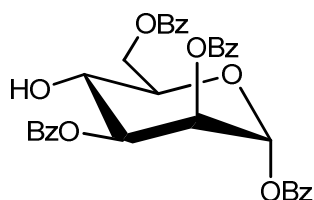
3. EXPERIMENTAL

3.1 General Procedures

Melting points were determined on a Griffin melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 141 polarimeter; $[\alpha]_D$ values are given in units of $10^{-1} \text{ deg cm}^2 \text{ g}^{-1}$. ^1H , ^{13}C and ^{31}P NMR spectra were recorded with Bruker AVANCE 500 MHz spectrometer, unless otherwise indicated. Chemical shifts (δ in ppm) are given relative to those for Me_4Si (for ^1H and ^{13}C) and external aq. 85% H_3PO_4 (for ^{31}P); all J values are given in Hz. ES mass spectra were recorded with a Mariner™ Biospectrometry Workstation and Agilent spectrometer. High resolution mass spectra were recorded on a Bruker microTOF. Thin layer chromatography (TLC) was performed on Kieselgel 60 F_{254} (silica gel) aluminium

plates (Merck) with A, toluene-ethyl acetate (9:1); B, toluene-methanol (7:3); C, toluene-ethyl acetate (95:5); D, toluene-ethyl acetate (7:3); E, toluene-ethyl acetate (75:25); F, petroleum ether (40-60 °C)-ethyl acetate (6:4); G, toluene-ethyl acetate (8:2); H, dichloromethane-methanol (9:1) as developers, followed by detection under UV light and/or by charring, which was carried out with sulphuric acid-water-ethanol (15:85:5) for protected compounds or a mixture of orcinol containing sulphuric acid-ethanol-water (10:75:5) for final deprotected compounds. Flash column chromatography (FCC) was performed on Kieselgel 60 (0.040-0.063 mm) (Merck), unless otherwise indicated. Dichloromethane, acetonitrile, pyridine and toluene were freshly distilled from CaH₂. Petroleum ether refers to that with boiling fraction 40-60 °C, unless otherwise stated. Solutions worked up were concentrated under reduced pressure at < 40 °C.

1,2,3,6-Tetra-*O*-benzoyl- α -D-mannopyranose 21



D-Mannose (3.6 g, 20 mmol) was dissolved in pyridine (25 ml) and the solution was stirred and cooled to -40 °C. Benzoyl chloride (9.27 ml, 80 mmol) in pyridine (15 ml) was added dropwise over 40 minutes. Once the addition was complete the mixture was allowed to warm to room temperature. After 1 h TLC (Solvent A) showed that the reaction was complete. The reaction was quenched by addition of water (100

ml), the mixture was stirred for 1 h, prior it was diluted with CH₂Cl₂ (200 ml). The organic layer was separated and washed successively with 1M HCl, water and saturated aq. NaHCO₃, dried (MgSO₄) and concentrated. The residue was dissolved in ethanol (100 ml), and kept at 4 °C overnight. The crystals were filtered off and mother liquor was removed. Dried crystals were dissolved in ethyl acetate with heating and *n*-hexane was added slowly over 10 min. The mixture was cooled and was placed at 4 °C for 16 h. The mother liquid was removed and the crystals were dried. The crystals were desired protected compound **21** (5.72 g, 9.58 mmol, 48%).

Mp 184-185 °C; literature data ^[99] mp 183-184 °C.

$[\alpha]_D^{20} +19.5$ (c 1, CHCl₃); literature data ^[99] $[\alpha]_D^{20} +41$ (c 1, CHCl₃).

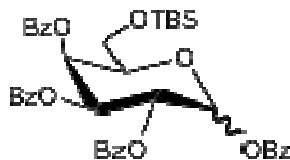
¹H NMR (CDCl₃): δ_H 3.24 (1H, d, $J_{4,OH}$ 5.0, 4-OH), 4.25(1H, ddd, $J_{5,6a}$ 2.2, H-5), 4.38 (1H, dt, $J_{3,4} = J_{4,5} = 10.2$, H-4), 4.52 (1H, dd, $J_{6a,6b}$ 12.4, H-6a), 5.02 (1H, dd, $J_{5,6b}$ 2.8, H-6b), 5.82 (2H, m, H-2 & H-3), 6.56 (1H, d, $J_{1,2}$ 1.5, H-1) and 7.24-8.20 (20H, m, 4 x Ph).

¹³C NMR (CDCl₃): δ_C 62.81 (C-6), 65.38 (C-4), 69.39 (C-2), 71.93 (C-3), 73.76 (C-5), 91.46 (C-1), 128.42-133.54 (Ph) and 164.76-165.12(C=O).

High resolution ES-MS(+): found m/z 614.2012 [M + NH₄]⁺ (C₃₄H₂₈O₁₀ requires M:

596.1682; C₃₄H₃₂NO₁₀⁺ requires m/z 614.2021) and 619.1564 [M + Na]⁺ (C₃₄H₂₈NaO₁₀⁺ requires m/z 619.1575).

1,2,3,4-Tetra-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl- α,β -D-galactopyranose **22**



D-Galactose (1 g, 5.56 mmol) was dissolved in pyridine (15 ml), and the solution was cooled to 0 °C. *tert*-Butyldimethylsilyl chloride (1.09 g, 7.22 mmol) was added, and the reaction was monitored by TLC (Solvent B). After 2 h, benzoyl chloride (5.15 ml, 44.4 mmol) was added dropwise to the cooled solution. After a further 14 h, TLC (Solvent C) showed complete benzoylation. Water (25 ml) was added and the reaction mixture was left to stir for 1 h. The mixture was diluted with CH₂Cl₂, and washed successively with 1M HCl, water and saturated aq. NaHCO₃, dried (MgSO₄) and concentrated. FCC [toluene-ethyl acetate (95:5)] of the residue gave compound **22** (3.25 g, 4.576 mmol, 82 %) as a white foam.

$[\alpha]_D^{20} +34.5$ (*c* 1, CHCl₃).

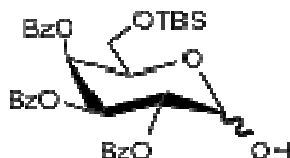
¹H NMR (CDCl₃): δ_H -0.07, -0.06, -0.02, 0.00 (4 x s, CH₃Si), 0.83, 0.85 (2 x s, *tert*-Bu), 3.80-3.97 (m, H-6a & H-6b), 4.30 (ddd, $J_{5\beta,6a\beta}$ 1.5, $J_{5\beta,6b\beta}$ 5.5, H-5 β), 4.55 (dt, $J_{5\alpha,6\alpha}$ 6.9, H-5 α), 5.81 (dd, $J_{3\beta,4\beta}$ 3.5, H-3 β), 6.00 (dd, $J_{3\alpha,4\alpha}$ 3.7, H-3 α), 6.08 (dd, $J_{2\beta,3\beta}$ 10.3, H-2 β), 6.10 (dd, $J_{4\beta,5\beta}$ 0.9, H-4 β), 6.16 (dd, $J_{2\alpha,3\alpha}$ 10.4, H-2 α), 6.18 (dd, $J_{4\alpha,5\alpha}$ 1.1, H-4 α), 6.25 (d, $J_{1\beta,2\beta}$ 8.3, H-1 β), 6.93 (d, $J_{1\alpha,2\alpha}$ 3.6, H-1 α) and 7.20-8.30 (m, Ph); $\alpha:\beta$ = 3:4.

^{13}C NMR (CDCl_3): δ_{c} -5.72, -5.67 (CH_3Si), 18.01 (Me_3C), 25.63, 25.72 (Me_3C), 60.31 (C-6 β), 60.62 (C-6 α), 67.53, 67.98, 68.18, 68.82, 68.99, 61.91 (C-2 α , C-3 α , C-4 α , C-5 α , C-2 β & C-4 β), 71.80 (C-3 β), 74.85 (C-5 β), 90.69 (C-1 α), 93.22 (C-1 β), 128.16-134.60 (Ph) and 165.49-166.13 (C=O).

High resolution ES-MS(+): found m/z 733.2445 $[\text{M} + \text{Na}]^+$ ($\text{C}_{40}\text{H}_{42}\text{O}_{10}\text{Si}$ requires M : 710.2547; $\text{C}_{40}\text{H}_{42}\text{NaO}_{10}\text{Si}^+$ requires m/z 733.2439).

2,3,4-Tri-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl- α,β -D-galactopyranose

23



Method A

Compound **22** (2 g, 2.82 mmol) was dissolved in THF (5 ml) and 2M dimethylamine in tetrahydrofuran (20 ml, 40 mmol) was added. This was stirred with monitoring by TLC (Solvent C). After 12 h (**22** was not consumed completely), the mixture was

concentrated to dryness. FCC [toluene-ethyl acetate (98:2→95:5)] of the residue yielded the hemiacetal **23** (1 g, 1.65 mmol, 59 %).

Method B

Acetic acid (81 μ l, 1.41 mmol) was added dropwise to a stirring solution of ethylenediamine (190 μ l, 2.85 mmol) in THF (25 ml). This instantly formed a white precipitate. The protected derivative **22** (1 g, 1.41 mmol) was added and the reaction mixture was left for 30 h. TLC (Solvent C) showed the reaction was complete. Water was added and, after 10 min, the reaction mixture was diluted with CH_2Cl_2 and washed successively with 1M HCl, water and saturated aq. NaHCO_3 , dried (MgSO_4) and concentrated. FCC [toluene-ethyl acetate (98:2)] of the residue yielded the hemiacetal **23** (650 mg, 1.07 mmol, 75 %) as an amorphous solid.

$[\alpha]_D^{20} +14.2$ (c 1, CHCl_3).

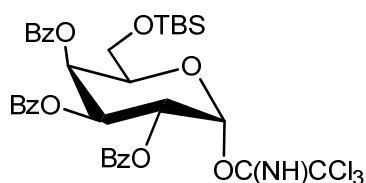
^1H NMR (CDCl_3): δ_{H} -0.13, -0.08, 0.00 (3 x s, CH_3Si), 0.75, 0.77 (2 x s, *tert*-Bu), 3.64-3.81 (m, H-6 α & H-6 β), 3.97 (ddd, $J_{5\beta,6\alpha\beta}$ 6.8, $J_{5\beta,6\beta\beta}$ 7.9, H-5 β), 4.50 (dt, $J_{5\alpha,6\alpha}$ 7.2, H-5 α), 4.92 (d, $J_{1\beta,2\beta}$ 7.9, H-1 β), 5.47 (dd, $J_{2\beta,3\beta}$ 10.3, H-2 β), 5.56 (dd, $J_{2\alpha,3\alpha}$ 10.6, H-2 α), 5.64 (dd, $J_{3\beta,4\beta}$ 3.5, H-3 β), 5.73 (d, $J_{1\alpha,2\alpha}$ 3.6, H-1 α), 5.89 (dd, $J_{4\beta,5\beta}$ 0.8, H-4 β), 5.92 (dd, $J_{4\alpha,5\alpha}$ 1.1, H-4 α), 5.97 (dd, $J_{3\alpha,4\alpha}$ 3.4, H-3 α), and 7.15-8.05 (m, Ph); $\alpha:\beta$ = 5:2.

^{13}C NMR (CDCl_3): δ_{C} -5.68, -5.58 (CH_3Si), 18.01 (Me_3C), 25.81 (Me_3C), 60.59 (C-6 β), 61.25 (C-6 α), 67.76, 68.28, 68.97, 69.43, 69.84 (C-2 α , C-3 α , C-4 α , C-5 α , C-2 β & C-

4 β), 71.19 (C-3 β), 74.23 (C-5 β), 91.04 (C-1 α), 96.36 (C-1 β), 128.22-133.67 (Ph) and 165.49-166.13 (C=O).

High resolution ES-MS(+): found m/z 629.2183 [$M + Na$] $^+$ ($C_{33}H_{38}O_9Si$ requires M : 606.2285; $C_{33}H_{38}NaO_9Si^+$ requires m/z 629.2177).

2,3,4-Tri-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl- α -D-galactopyranosyl trichloroacetimidate **24**



Compound **23** (583 mg, 0.981 mmol) was dissolved in CH_2Cl_2 (4 ml), the solution cooled to 0 °C while trichloroacetonitrile (964 μ l, 9.61 mmol) and Cs_2CO_3 (cat.) were respectively added. After 1 h monitoring by TLC (Solvent C) showed one product and no starting material left. The mixture was diluted with CH_2Cl_2 , filtered through celite and concentrated to dryness. FCC [toluene-ethyl acetate (98:2→95:5)] of the residue gave the trichloroacetimidate **24** (379 mg, 0.625 mmol, 64 %) as an amorphous solid.

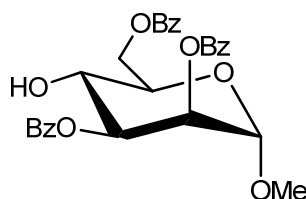
$[\alpha]_D^{20} +42.8$ (c 1, CHCl₃).

¹H NMR (CDCl₃): δ_H 0.00, 0.05 (6H, 2 x s, CH₃Si), 0.90 (9H, s, *tert*-Bu), 3.84 (1H, dd, $J_{5,6a}$ 7.7, H-6a), 3.90 (1H, dd, $J_{6a,6b}$ 10.1, H-6b), 4.59 (1H, ddd, $J_{5,6b}$ 6.1, H-5), 5.96 (1H, dd, $J_{2,3}$ 10.6, H-2), 6.14 (1H, dd, $J_{3,4}$ 3.3, H-3), 6.18 (1H, dd, $J_{4,5}$ 1.2, H-4), 6.94 (1H, d, $J_{1,2}$ 3.7, H-1), 7.24-8.18 (15H, m, Ph) and 8.69 (1H, s, NH).

¹³C NMR (CDCl₃): δ_C -5.65, -5.59 (CH₃Si), 18.01 (Me₃C), 25.74 (Me₃C), 60.82 (C-6), 68.21, 68.59, 72.22 (C-2, C-3, C-4 & C-5), 93.93 (C-1), 128.28-133.48(Ph) and 165.49-166.13 (C=O).

High resolution ES-MS(+): found m/z 605.2132 [M - CNHCCl₃]⁺ (C₃₅H₃₈Cl₃NO₁₀Si requires M: 749.1381; C₃₃H₃₇O₈Si⁺ requires m/z 605.2212).

Methyl 2,3,6-tri-O-benzoyl- α -D-mannopyranoside 25



Methyl α -D-mannopyranoside (1.5 g, 7.7 mmol) was dissolved in pyridine (30 ml) and the solution was cooled to -40 °C. Benzoyl chloride (3.48 g, 24.7 mmol) in pyridine (20 ml) was added dropwise over 1 h and the mixture was left for 20 h at rt. Water was added, the mixture was stirred for 2 h prior it was diluted with CH₂Cl₂.

The organic layer was washed successively with 1M HCl, water and saturated aq. NaHCO₃, dried (MgSO₄) and concentrated. FFC [toluene-ethyl acetate (99:1→90:10)] of the residue gave the protected monosaccharide **25** (2.75 g, 5.43 mmol, 71 %) as a white foam.

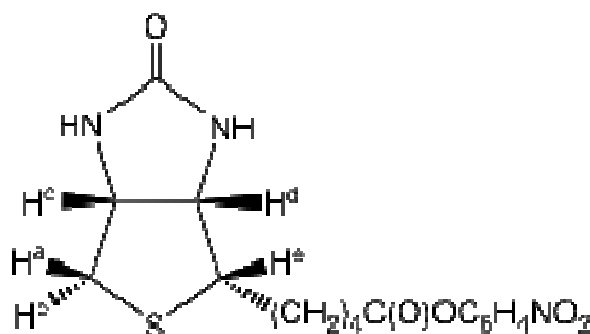
$[\alpha]_D^{20} +19.3$ (c 1, CHCl₃).

¹H NMR (CDCl₃): δ_H 3.50 (3H, s, OMe), 4.12 (1H, ddd, *J*_{5,6a} 2.8, H-5), 4.31 (1H, t, *J*_{3,4} = *J*_{4,5} = 9.5), 4.67 (1H, dd, *J*_{6a,6b} 12.1, H-6a), 4.93 (1H, dd, *J*_{5,6b} 3.9, H-6b), 4.96 (1H, d, *J*_{1,2} = 1.0, H-1), 5.63-5.67 (2H, m, H-2 & H-3) and 7.20-8.17 (15H, m, Ph).

¹³C NMR (CDCl₃): δ_C 55.39 (OMe), 63.40 (C-6), 63.34 (C-4), 70.47 (C-2), 71.19 (C-5), 72.63 (C-3), 98.70 (C-1), 128.25-133.41 (Ph) and 166.73-166.96 (C=O).

High resolution ES-MS(+): found *m/z* 507.1653 [M + H]⁺ (C₂₈H₂₆O₉ requires M: 506.1577; C₂₈H₂₇O₉⁺ requires *m/z* 507.1650) and 529.1483 [M + Na]⁺ (C₂₈H₂₇NaO₉⁺ requires *m/z* 529.1469).

D-Biotin *p*-nitrophenyl ester 26



D-Biotin (200 mg, 0.82 mmol) was added to pyridine (5 ml) under stirring. *p*-Nitrophenyl trifluoroacetate (731 mg, 3.11 mmol) and *p*-nitrophenol (103 mg, 0.74 mmol) were added to the suspension. The mixture was stirred at 55 °C for 2 h and evaporated to dryness. The dried residue was collected and washed with Et₂O (3 times) to remove excessive *p*-nitrophenol. The filtrate was recrystallised from ethanol, the crystals were harvested and dried under vacuum. This gave the biotin ester **26** (210 mg, 0.57 mmol, 72 %) as crystals.

Mp 164-165 °C.

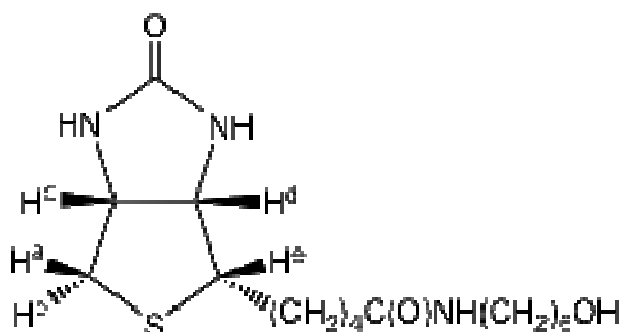
$$[\alpha]_D^{20} +22.1 \text{ (c 1, MeOH).}$$

¹H NMR (CDCl₃): δ_H 1.37-1.45 (2H, m, CH₂), 1.52-1.71 (4H, m, 2 x CH₂), 2.49 (2H, t, *J*_{a,b} 7.4, CH₂CO), 2.65 (1H, br d, *J*_{a,b} 13.0, H^b), 2.80 (1H, dd, *J*_{a,c} 5.0, H^a), 3.06 (1H, m, H^e), 4.24 (1H, dd, *J*_{c,d} 7.8, *J*_{d,e} 4.5, H^d), 4.44 (1H, m, H^c) and 7.13-8.14 (4H, m, C₆H₄).

^{13}C NMR $[(\text{CD}_3)_2\text{SO}]$: δ_{c} 24.15, 27.85, 27.94 (3 x CH_2), 33.25 (CH_2CO), 39.81 (C^{ab}), 55.27 (C^{e}), 59.23 (C^{c}), 61.02 (C^{d}), 123.16, 125.24, 144.95, 155.37 (C_6H_4), 162.73 (NHCONH) and 171.14 (CH_2COO).

High resolution ES-MS(+): found m/z 366.1110 $[\text{M} + \text{H}]^+$ ($\text{C}_{16}\text{H}_{19}\text{N}_3\text{O}_5\text{S}$ requires M : 365.1045; $\text{C}_{16}\text{H}_{20}\text{N}_3\text{O}_5\text{S}^+$ requires m/z 366.1118).

6-*N*-Biotinylaminohexanol **27**



Compound **26** (500 mg, 1.37 mmol) and 6-aminohexanol (161 mg, 1.37 mmol) were added to DMSO (5 ml) and the solution was stirred for 20 h. The reaction was stopped by pouring the solution into a mixture of acetone (10 ml) and Et_2O (16 ml). The resultant suspension was placed in the fridge overnight. The precipitate was

filtered off and washed with Et₂O (4x). This gave the modified biotin derivative **27** (392 mg, 1.01 mmol, 80%) as an amorphous solid.

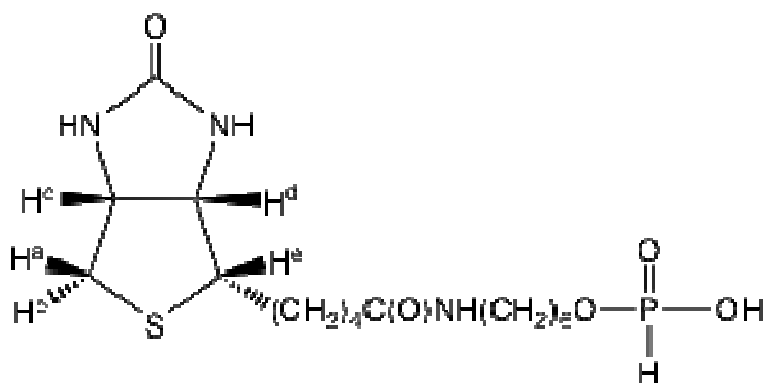
$[\alpha]_D^{20} +12.6$ (c 1, MeOH).

¹H NMR [(CD₃)₂SO]: δ_H 1.20 -1.65 (14H, m, 7 x CH₂), 2.04 (2H, t, *J* 7.4, CH₂CO), 2.58 (1H, br d, *J*_{a,b} 12.4, H^b), 2.82 (1H, dd, *J*_{a,c} 5.1, H^a), 3.00 (2H, q, *J* 6.5, CH₂N), 3.09 (1H, m, H^e), 3.37 (2H, dt, *J*_{H,H} 6.5, *J*_{H,OH} 5.2, CH₂OH), 4.13 (1H, m, H^d), 4.31 (1H, m, H^c), 4.36 (1H, t, *J*_{H,OH} 5.2, CH₂OH), 6.38 (1H, br, NH) 6.45 (1H, br, NH) and 7.75 (1H, t, *J* 5.5 CH₂NH).

¹³C NMR [(CD₃)₂SO]: δ_C 25.23, 25.32, 26.32, 28.01, 28.19, 29.22 (6 x CH₂), 32.47 (CH₂CH₂OH), 35.18 (CH₂CO), 38.32 (CH₂N), 39.82 (C^{ab}), 55.42 (C^e), 59.13 (C^c), 60.63 (CH₂OH), 61.00 (C^d), 162.68 (NHCONH) and 171.75 (CH₂CONH).

High resolution ES-MS(+): found *m/z* 344.2006 [M + H]⁺ (C₁₆H₂₉N₃O₃S requires M: 343.1930; C₁₆H₃₀N₃O₃S⁺ requires *m/z* 344.2002).

6-*N*-Biotinylaminohexyl hydrogenphosphonate **28**



Compound **27** (100 mg, 0.29 mmol) was dissolved in a mixture of DMF (5 ml) and pyridine (60 μ l). A solution of salicyl chlorophosphate (76 mg, 0.38 mmol) in DMF (5ml) was added dropwise over 10 min to the stirred solution. After a further 10 min, 1M TEAB buffer (0.5 ml) was added and the mixture left for 15 min. Et₂O (55 ml) was then added and the mixture was placed in the fridge for 16 h. The precipitate that had formed was filtered off, washed with Et₂O (3 x 30 ml) and CH₂Cl₂ (3 x 30 ml) and dried under vacuum. This gave the biotinylaminohexyl hydrogenphosphonate **28** (106 mg, 0.261 mmol, 90 %) as an amorphous solid.

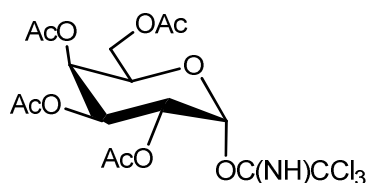
$[\alpha]_D^{20} +19.1$ (*c* 1, MeOH).

¹H NMR (CD₃OD): δ_H 1.34-1.77 (14H, m, 7 x CH₂), 2.28 (2H, t, *J* 7.4, CH₂CO), 2.70 (1H, d, *J*_{a,b} 12.1, H^b), 2.92 (1H, dd, *J*_{a,c} 4.0, H^a), 3.15-3.23 (3H, m, H^e & CH₂N), 4.01 (2H, dt, *J*_{H,P} 8.4, *J*_{H,H} 6.5, CH₂OP), 4.30 (1H, dd, *J*_{d,e} 4.5, H^d), 4.49 (1H, dd, *J*_{c,d} 7.8, H^c) and 6.76 (1H, d, *J*_{H,P} 680.8, HP).

^{13}C NMR (CD_3OD): δ_{c} 26.36, 26.95, 27.49, 29.53, 29.79, 30.32 (6 x CH_2), 31.45 (d, $^3J_{\text{C,P}}$ 6.5, $\text{CH}_2\text{CH}_2\text{OP}$), 36.84 (CH_2CO), 40.25 (CH_2N), 41.04 (C^{ab}), 57.03 (C^{e}), 61.68 (C^{c}), 63.45 (C^{d}), 66.31 (d, $^2J_{\text{C,P}}$ 5.1, CH_2OP), 166.13 (NHCONH) and 176.02 (CH_2CONH).

High resolution ES-MS(+): found m/z 408.1722 $[\text{M} + \text{H}]^+$ ($\text{C}_{16}\text{H}_{30}\text{N}_3\text{O}_5\text{PS}$ requires M : 407.1644; $\text{C}_{16}\text{H}_{31}\text{N}_3\text{O}_5\text{PS}^+$ requires m/z 408.1717).

2,3,4,6-Tetra-*O*-acetyl- α -D-galactopyranosyl trichloroacetimidate 29



Penta-*O*-acetyl- β -D-galactopyranose (1.2 g, 3.07 mmol) was dissolved in MeCN (10 ml), to this mixture 2M dimethylamine in tetrahydrofuran (8 ml, 16 mmol) was added and the solution left at rt for 1 h. Monitoring by TLC (Solvent D) showed one major product (thought to be 2,3,4,6-tetra-*O*-acetyl-D-galactopyranose). The solution was evaporated to dryness, the residue was dissolved in CH_2Cl_2 , washed with water (3 x 50 ml), dried (MgSO_4) and concentrated. The residue was dissolved in CH_2Cl_2 (10 ml), the solution was cooled to 0 °C and trichloroacetonitrile (3.07 ml, 30.7 mmol) and Cs_2CO_3 (cat.) were added under stirring. After 2 h, monitoring by TLC (Solvent D) showed the reaction was complete. The mixture was diluted with CH_2Cl_2 ,

filtered through a Celite pad and the filtrate was concentrated. FCC [toluene-ethyl acetate (1:99→12:88)] of the residue gave the trichloroacetimidate **29** (1.21 g, 2.45 mmol, 80 %) as an amorphous solid.

$[\alpha]_D^{20} +23.6$ (c 1, CHCl₃).

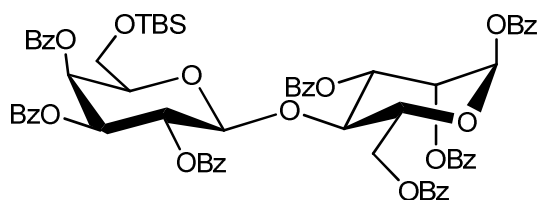
¹H NMR (CDCl₃): δ_H 2.03-2.06 (9H, m, 3 x Ac), 2.19 (3H, s, Ac), 4.11 (1H, dd, $J_{6a,6b}$ 11.3, $J_{6a,5}$ 6.7, H-6a), 4.19 (1H, dd, $J_{6b,5}$ 6.7, H-6b), 4.46 (1H, dt, $J_{5,4}$ 0.7, H-5), 5.37 (1H, dd, $J_{2,3}$ 10.8, H-2), 5.44 (1H, dd, $J_{3,4}$ 3.4, H-3), 5.58 (1H, dd, H-4), 6.63 (1H, d, $J_{1,2}$ 3.4, H-1) and 8.69 (1H, s, NH).

¹³C NMR (CDCl₃): δ_C 20.50, 20.58 (CH₃), 61.20 (C-6), 66.85 (C-2), 67.32 (C-4), 67.45 (C-3), 68.94 (C-5), 90.72 (CCl₃), 93.48 (C-1), 160.88 (C=NH) and 169.91-170.23 (C=O).

Low resolution ES-MS(+): found m/z 514.00 [M + Na]⁺ (C₁₆H₂₀Cl₃N₃O₁₀ requires

M:491.02; C₁₆H₂₀Cl₃N₃NaO₁₀⁺ requires m/z 514.0045).

1,2,3,6-Tetra-*O*-benzoyl-4-*O*-(2,3,4-tri-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl- β -D-galactopyranosyl)- α -D-mannopyranose **30**



A mixture of compounds **24** (650 mg, 0.865 mmol) and **21** (430 mg, 0.721 mmol) was dissolved in freshly distilled CH_2Cl_2 (3 ml), before freshly activated molecular sieves 4\AA (500 mg) were added. This suspension was cooled to $-30\text{ }^\circ\text{C}$ under stirring and trimethylsilyl trifluoromethanesulfonate (39 μl , 0.216 mmol) was injected through the septa. Monitoring by TLC (Solvent C) showed that after 30 minutes the glycosyl donor **24** was consumed. The reaction was stopped by adding triethylamine (100 μl) in CH_2Cl_2 (1ml) and left for 10 min. The mixture was diluted with CH_2Cl_2 , filtered through a Celite pad to remove solids and the filtrate was concentrated. FCC [toluene-ethyl acetate (100:0 \rightarrow 95:5)] of the residue gave the protected disaccharide **30** (738 mg, 0.623 mmol, 72 %) as an amorphous solid.

$[\alpha]_D^{20} +36.8$ (c 1, CHCl_3).

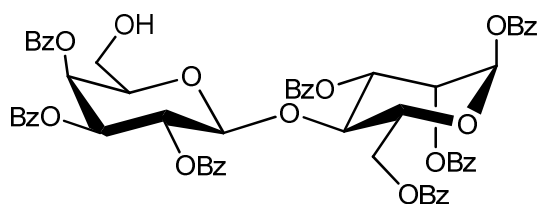
^1H NMR (CDCl_3): δ_{H} -0.10 (3H, s, CH_3Si), -0.01 (3H, s, CH_3Si), 0.93 (9H, s, *tert*-Bu), 3.40-3.48 (2H, m, H-6b' & H-6a'), 3.78 (1H, ddd, $J_{5',6a'}$ 5.5, $J_{5',6b'}$ 9.1, H-5'), 4.49 (1H, ddd, $J_{5,6a}$ 2.8, H-5), 4.73 (1H, dd, $J_{6a,6b}$ 12.2, H-6a), 4.87 (1H, dd, $J_{5,6b}$ 2.0, H-6b), 4.89 (1H, t, $J_{3,4} = J_{4,5} = 9.6$, H-4), 5.22 (1H, d, $J_{1',2'}$ 7.9 H-1'), 5.69 (1H, ddd, $J_{3',4'}$ 3.3, H-3'), 5.93 (1H,

dd, $J_{2',3'}$ 10.4, H-2'), 5.98 (1H, dd, $J_{4',5'}$ 0.8, H-4'), 6.07 (1H, dd, $J_{2,3}$ 3.4, H-2), 6.22 (1H, dd, H-3), 6.72 (1H, d, $J_{1,2}$ 2.0, H-1) and 7.38-8.40 (35H, m, Ph).

^{13}C NMR (CDCl_3): δ_{c} -5.80, -5.66 (2 x CH_3Si), 18.17 (Me_3C), 25.63 (Me_3C), 59.32 (C-6'), 61.77 (C-6), 67.01 (C-4'), 69.39 (C-2), 70.36 (C-2'), 70.60 (C-3), 71.61 (C-5), 71.96 (C-3'), 73.40 (C-4), 73.56 (C-5'), 91.30 (C-1), 101.72 (C-1'), 128.21-134.13 (Ph) and 163.97-165.51 (C=O).

High resolution ES-MS(+): found m/z 1207.3760 $[\text{M} + \text{Na}]^+$ ($\text{C}_{67}\text{H}_{64}\text{O}_{18}\text{Si}$ requires M : 1184.3862; $\text{C}_{67}\text{H}_{64}\text{NaO}_{18}\text{Si}^+$ requires m/z 1207.3754).

1,2,3,6-Tetra-*O*-benzoyl-4-*O*-(2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl)- α -D-mannopyranose **31**



Acetonitrile (2 ml) was used to dissolve the disaccharide **30** (500 mg, 0.423 mmol), and the solution was cooled to 0 °C. A mixture of 40 % aq. HF and MeCN (1:1, 2 ml) was added under stirring. This gave a resultant concentration of 10 % HF in the reaction mixture. The reaction was run for 3 h and monitored by TLC (Solvent E). To stop the reaction saturated aq. NaHCO_3 was added dropwise to the mixture until the

bubbling stopped. The mixture was diluted with CH₂Cl₂ (150 ml), washed with water (5 x 40 ml), dried (MgSO₄) and concentrated. A solution of the residue in CH₂Cl₂ was filtered through a silica gel pad and concentrated to dryness. This gave the disaccharide **31** (430 mg, 0.402 mmol, 95 %) as an amorphous solid.

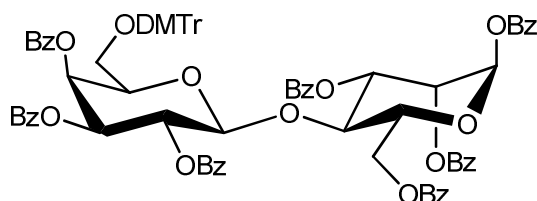
¹H NMR (CDCl₃): δ_H 3.09 (1H, dd, *J*_{6a',6b'} 11.9 H-6a'), 3.20 (1H, dd, *J*_{5',6b'} 6.3, H-6b') 3.65 (1H, dd, *J*_{5',6a'} 7.6, H-5'), 4.31 (1H, ddd, *J*_{5,6a} 2.9, H-5), 4.54 (1H, dd, *J*_{6a,6b} 12.3, H-6a), 4.67 (1H, dd, *J*_{5,6b} 1.7, H-6b), 4.71 (1H, t, *J*_{3,4} = *J*_{4,5} = 9.4, H-4), 5.04 (1H, d, *J*_{1',2'} 7.9 H-1'), 5.46 (1H, dd, *J*_{2',3'} 10.4, H-3'), 5.64 (1H, d, *J*_{3',4'} 3.3, H-4'), 5.87 (2H, m, H-2 & H-2'), 6.06 (1H, dd, *J*_{2,3} 3.4, H-3), 6.56 (1H, d, *J*_{1,2} 2.3, H-1) and 7.12 -8.24 (35H, m, Ph).

¹³C NMR (CDCl₃): δ_C 59.89 (C-6'), 61.92 (C-6), 68.53 (C-4'), 69.63 (C-2), 70.12(C-3), 70.18 (C-2'), 71.59 (C-5), 71.83 (C-3'), 73.56 (C-4), 74.13 (C-5') 91.32 (C-1), 101.49 (C-1'), 128.25-134.02 (Ph) and 164.06-166.67 (C=O).

High resolution ES-MS(+): found *m/z* 1093.2895 [M + Na]⁺ (C₆₁H₅₀O₁₈ requires M: 1070.2997; C₆₁H₅₀NaO₁₈⁺ requires *m/z* 1093.2889).

1,2,3,6-Tetra-*O*-benzoyl-4-*O*-[2,3,4-tri-*O*-benzoyl-6-*O*-(*p,p'*-dimethoxytrityl)- β -D-galactopyranosyl]- α -D-mannopyranose **32**

(Method A)



Compound **31** (600 mg, 0.56 mmol) was dissolved in pyridine (3 ml), and the solution was cooled to 0 °C. *p,p'*-Dimethoxytriphenylmethyl chloride (474 mg, 1.4 mmol) was added and the reaction mixture was left for 15 h at rt. Monitoring by TLC (Solvent F) showed the reaction was complete. The mixture was diluted with CH₂Cl₂, washed with water (2 x 50 ml), dried by filtration through cotton wool, concentrated and toluene was evaporated off the residue. FCC [toluene-ethyl acetate (80:20→70:30)] of the residue provided the protected disaccharide **32** (630 mg, 0.46 mmol, 82 %). Compound **12** was later prepared in an alternative way from the disaccharide derivative **39**.

$[\alpha]_D^{20} +62.7$ (*c* 1, CHCl₃).

¹H NMR (CDCl₃): δ_H 3.02 (1H, t, $J_{5',6a'} = J_{6a',6b'} = 9.0$, H-6a'), 3.15 (1H, dd, $J_{5',6b'}$ 5.1, H-6b'), 3.56, 3.57 (6H, 2 x s, 2 x OMe), 3.73 (1H, br dd, H-5'), 4.07 (1H, ddd, $J_{5,6a}$ 1.8, H-5), 4.44 (1H, dd, $J_{6a,6b}$ 12.1, H-6a), 4.49 (1H, dd, $J_{5,6b}$ 3.1, H-6b), 4.56 (1H, t, $J_{3,4} = J_{4,5} = 9.7$, H-4), 4.88 (1H, d, $J_{1',2'}$ 7.8, H-1'), 5.40 (1H, dd, $J_{2',3'}$ 10.3, H-3'), 5.55 (1H, dd, H-2'),

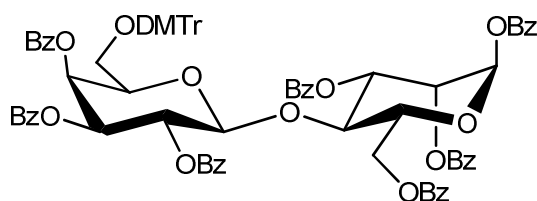
5.73 (1H, dd, H-2), 5.79 (1H, dd, $J_{2,3}$ 3.3, H-3), 5.91 (1H, br d, $J_{3',4'}$ 2.9, H-4'), 6.40 (1H, d, $J_{1,2}$ 2.0, H-1) and 6.46-8.20 (48H, m, C₆H₄ & Ph).

¹³C NMR (CDCl₃): δ_c 55.03 (OMe), 59.52 (C-6'), 62.07 (C-6), 67.52 (C-4'), 69.58 (C-2), 70.10 (C-3), 70.24 (C-2'), 71.95 (C-5), 72.10 (C-3'), 72.56 (C-5'), 72.65 (C-4), 86.25 (Ar₃C), 91.19 (C-1), 101.39 (C-1'), 113.00, 126.75-135.65, 143.97, 158.27, 158.39 (C₆H₆ and Ph) and 163.96-165.67 (C=O).

High Resolution ES-MS(+): found m/z 1395.4202 [M+Na]⁺ (C₈₂H₆₈O₂₀ requires M: 1372.4304; C₈₂H₆₈NaO₂₀⁺ requires m/z 1395.4196).

1,2,3,6-Tetra-*O*-benzoyl-4-*O*-[2,3,4-tri-*O*-benzoyl-6-*O*-(*p,p'*-dimethoxytrityl)- β -D-galactopyranosyl]- α -D-mannopyranose **32**

(Method B)



A solution of HCl in MeOH [prepared at 0 °C from acetyl chloride (0.2 ml) and methanol (5 ml)] was added to a solution of compound **39** (600 mg, 0.55 mmol) in CH₂Cl₂ (2 ml) and the mixture was stirred at rt for 20 h. Checking by TLC (solvent H) showed that there was one major product, thought to be the deacetylated

compound. The reaction mixture was diluted with CH_2Cl_2 and washed successively with saturated aq. NaHCO_3 (twice) and water (twice), dried by filtration through cotton wool and concentrated. The residue was dissolved in pyridine (10 ml), *p,p'*-dimethoxytriphenylmethyl chloride (560 mg, 1.65 mmol) was added and the solution was left stirring at rt for 20 h. Benzoyl chloride (510 μl , 4.4 mmol) was then added at -10°C and the mixture was stirred at rt for 15 h. TLC (solvent A) showed that the benzoylation had been completed. The reaction mixture was diluted with CHCl_3 and washed successively with saturated aq. NaHCO_3 (twice) and water (twice), dried by filtration through cotton wool and concentrated. FFC [toluene-ethyl acetate (100:0 \rightarrow 90:10)] of the residue gave the disaccharide **32** (546 mg, 0.398 mmol, 91%) as an a amorphous solid.

$[\alpha]_D^{20} +62.7$ (c 1, CHCl_3).

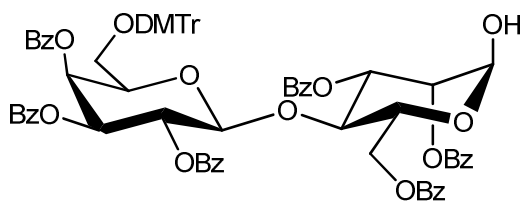
^1H NMR (CDCl_3): δ_{H} 3.02 (1H, t, $J_{5',6a'} = J_{6a',6b'} = 9.0$, H-6a'), 3.15 (1H, dd, $J_{5',6b'} = 5.1$, H-6b'), 3.56, 3.57 (6H, 2 x s, 2 x OMe), 3.73 (1H, br dd, H-5'), 4.07 (1H, ddd, $J_{5,6a} = 1.8$, H-5), 4.44 (1H, dd, $J_{6a,6b} = 12.1$, H-6a), 4.49 (1H, dd, $J_{5,6b} = 3.1$, H-6b), 4.56 (1H, t, $J_{3,4} = J_{4,5} = 9.7$, H-4), 4.88 (1H, d, $J_{1',2'} = 7.8$, H-1'), 5.40 (1H, dd, $J_{2',3'} = 10.3$, H-3'), 5.55 (1H, dd, H-2'), 5.73 (1H, dd, H-2), 5.79 (1H, dd, $J_{2,3} = 3.3$, H-3), 5.91 (1H, br d, $J_{3',4'} = 2.9$, H-4'), 6.40 (1H, d, $J_{1,2} = 2.0$, H-1) and 6.46-8.20 (48H, m, C_6H_4 & Ph).

^{13}C NMR (CDCl_3): δ_{C} 55.03 (OMe), 59.52 (C-6'), 62.07 (C-6), 67.52 (C-4'), 69.58 (C-2), 70.10 (C-3), 70.24 (C-2'), 71.95 (C-5), 72.10 (C-3'), 72.56 (C-5'), 72.65 (C-4), 86.25

(Ar₃C), 91.19 (C-1), 101.39 (C-1'), 113.00, 126.75-135.65, 143.97, 158.27, 158.39 (C₆H₆ and Ph) and 163.96-165.67 (C=O).

High Resolution ES-MS(+): found m/z 1395.4202 [M+Na]⁺ (C₈₂H₆₈O₂₀ requires M: 1372.4304; C₈₂H₆₈NaO₂₀⁺ requires m/z 1395.4196).

**2,3,6-Tri-*O*-benzoyl-4-*O*-[2,3,4-tri-*O*-benzoyl-6-*O*-(*p,p'*-dimethoxytrityl)-
β-D-galactopyranosyl]-α-D-mannopyranose **33****



Method A

Compound **32** (292 mg, 0.22 mmol) was dissolved in THF (2 ml), and 2M dimethylamine in tetrahydrofuran (2 ml, 4 mmol) was added. This was stirred at rt for 20 h with monitoring by TLC (Solvent G). The mixture was concentrated to dryness. FFC [toluene-ethyl acetate (90:10→80:20)] of the residue yielded the hemiacetal derivative **33** (200 mg, 0.14 mmol, 70 %) as an amorphous solid.

Method B

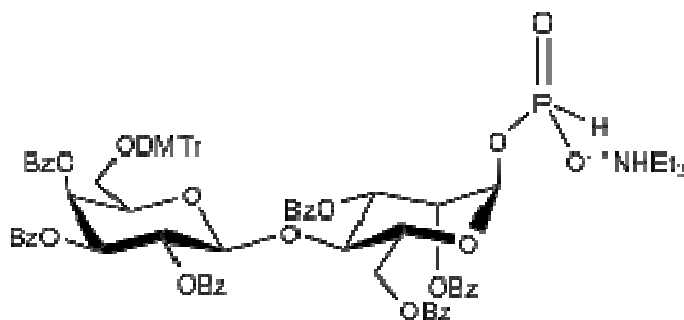
Acetic acid (101 μ l, 1.77 mmol) was added drop wise to a stirring solution of ethylenediamine (236 μ l, 3.54 mmol) in THF (4 ml). This instantly formed a white precipitate. The protected disaccharide **32** (250 mg, 1.77 mmol) was added and the reaction mixture and left for 40 h under stirring. TLC (Sovlent G) showed the reaction was complete. Water was added and, after 10 minutes, the mixture was diluted with CH_2Cl_2 and washed successively with 1M HCl, water and saturated aq. NaHCO_3 , dried (MgSO_4) and concentrated. FCC [toluene-ethyl acetate (85:15)] of the residue yielded the anomerically deprotected disaccrharide **33** (650 mg, 1.07 mmol, 75 %) as an amorphous solid.

$[\alpha]_D^{20} +56.1$ (c 1, CHCl_3).

^1H NMR (CDCl_3): δ_{H} 3.05 (1H, m, H-6a'), 3.17 (1H, dd, $J_{6a',6b'}$ 8.8, H-6b'), 3.55, 3.56 (6H, 2 x s, 2 x MeO), 3.66 (1H, br dd, $J_{5',6a'}$ 8.9, $J_{5',6b'}$ 5.1, H-5'), 4.20 (1H, dd, $J_{4,5}$ 9.8, $J_{5,6a}$ 2.3, H-5), 4.44 (2H, m, H-4 & H-6a), 4.52 (1H, dd, $J_{5,6b}$ 1.3, $J_{6a,6b}$ 12.0, H-6b), 4.84 (1H, d, $J_{1',2'}$ 7.9, H-1'), 5.21 (1H, d, $J_{1,2}$ 1.5, H-1), 5.38 (1H, dd, $J_{2',3'}$ 10.4, H-3'), 5.53 (2H, m, H-2 & H-2'), 5.73 (1H, dd, $J_{2,3}$ 3.3, $J_{3,4}$ 9.6, H-3), 5.87 (1H, br d, H-4') and 6.47-7.90 (43H, m, C_6H_4 & Ph).

^{13}C NMR (CDCl_3): δ_{C} 55.10 (OMe), 59.61 (C-6'), 62.67 (C-6), 67.58 (C-4'), 69.43 (C-5), 69.83 (C-3), 70.26 (C-2'), 71.19 (C-2), 71.85 (C-3'), 72.44 (C-5'), 73.07 (C-4), 86.27 (Ar_3C), 92.02 (C-1), 100.98 (C-1'), 113.03, 128.19-133.30, 144.08, 158.30, 158.38 (C_6H_4 & Ph) and 164.97-166.04 (C=O).

**2,3,6-Tri-*O*-benzoyl-4-*O*-[2,3,4-tri-*O*-benzoyl-6-*O*-(*p,p'*-dimethoxytrityl)-
 β -D-galactopyranosyl]- α -D-mannopyranosyl hydrogenphosphonate,
triethylammonium salt **34****



Method A

To a stirred solution of imidazole (157 mg, 2.30 mmol) in MeCN (3 ml) at 0 °C was added phosphorus trichloride (60 μ l, 0.68 mmol) and then triethylamine (334 μ l, 2.4 mmol). After 15 min, a solution of compound **33** (200 mg, 0.16 mmol) in MeCN (3 ml) was added dropwise during 15 min at 0 °C. The mixture was stirred for 30 min at rt and the reaction was quenched with 1M TEAB buffer solution (2-3 ml). The reaction mixture was diluted with CH₂Cl₂ (40 ml), washed successively with ice-water and cold 0.5M TEAB solution, dried by filtration through cotton wool and concentrated. FFC [CH₂Cl₂-methanol (9:1)] of the residue gave the derivative **34- α,β** (218 mg, 0.152 mmol, 95 %; an anomeric mixture at D-mannose residue) with a α/β ratio of 12:1 (determined using ¹H and ³¹P NMR data).

The prepared compound **34- α,β** was dissolved in a mixture of CH₂Cl₂ (4.2 ml) and methanol (0.9 ml). To this a solution of silver triflate (105 mg, 0.405 mmol) in toluene (2 ml) was added dropwise. After 40 min, the reaction was quenched by addition of cold 1M TEAB buffer solution. The mixture was diluted with CH₂Cl₂, washed with cold 0.5M TEAB buffer solution, dried by filtration through cotton wool and concentrated. The residue was filtered through a silica pad (to remove AgOTf) and concentrated. FFC [CH₂Cl₂-methanol (9:1)] of the residue gave the hydrogenphosphonate derivative **34** (106 mg, 0.076 mmol, 48 %; a pure α -anomer at D-mannose residue) as an amorphous solid.

Method B

The hemiacetal **33** (82.5 mg, 0.065 mmol) was dissolved in MeCN (2 ml), and to this solution triethylamine (600 μ l, 4.24 mmol) and salicyl chlorophosphite (89 mg, 0.437 mmol) were added under stirring. Monitoring by TLC (Solvent H) showed complete conversion of **33** to the H-phosphonate **34** after 1 h. The reaction mixture was diluted with CHCl₃, and washed successively by saturated aq. NaHCO₃, water and cold 0.5M TEAB buffer solution, dried by filtration through cotton wool and concentrated. FFC [CH₂Cl₂-methanol (9:1)] of the residue gave the hydrogenphosphonate derivative **34** (92 mg, 0.064 mmol, 98 %; a pure α -anomer at D-mannose residue) as an amorphous solid.

$[\alpha]_D^{20} +32.4$ (*c* 1, CHCl₃).

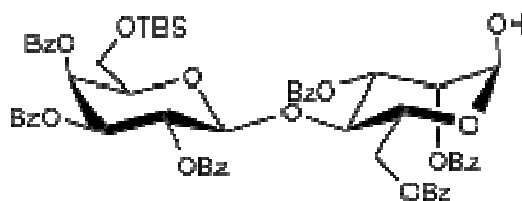
^1H NMR (CDCl_3): δ_{H} 1.30 (9H, t, 3 x MeCH_2), 3.09 (7H, m, H-6a' & MeCH_2), 3.24, (1H, dd, $J_{5',6b'}$ 5.0, $J_{6a',6b'}$ 8.6, H-6b'), 3.65, 3.66 (6H, 2 x s, 2 x MeO), 3.77 (1H, br dd, $J_{5',6a'}$ 9.3, H-5'), 4.31 (1H, dt, $J_{5,6}$ 2.8, H-5), 4.48 (1H, t, $J_{3,4} = J_{4,5} = 9.8$, H-4), 4.56 (2H, d, H-6a & H-6b), 4.89 (1H, d, $J_{1',2'}$ 7.9, H-1'), 5.47 (1H, dd, $J_{3',4'}$ 3.3, H-3'), 5.59 (1H, dd, $J_{2',3'}$ 10.5, H-2'), 5.68 (1H, dd, $J_{2,3}$ 3.3, H-2), 5.72 (1H, dd, $J_{1,2}$ 2.0, $J_{1,\text{P}}$ 9.0, H-1), 5.76 (1H, dd, H-3), 5.98 (1H, br d, H-4'), 7.02 (1H, d, $J_{\text{H,P}}$ 636.1, HP) and 6.53-8.05 (43H, m, C_6H_4 & Ph).

^{13}C NMR (CDCl_3): δ_{C} 8.47 (MeCH_2), 45.55 (MeCH_2), 55.05 (OMe), 59.55 (C-6'), 62.58 (C-6), 67.39 (C-4'), 69.83 (C-3), 70.05 (C-5), 70.26 (C-2'), 70.86 (d, $J_{\text{C,P}}$ 6.9, C-2), 71.99 (C-3'), 72.40 (C-5'), 72.78 (C-4), 86.27 (Ar_3C), 92.60 (d, $J_{\text{C,P}}$ 3.6, C-1), 100.98 (C-1'), 113.00, 126.72-130.30, 132.65-135.60 144.02, 158.28, 158.38 (C_6H_4 & Ph) and 164.97-166.24 (C=O)

^{31}P NMR (CDCl_3): δ_{P} 0.38.

High resolution ES-MS(-): found m/z 1331.3780 [$\text{M} - \text{Et}_3\text{N} - \text{H}$] $^-$ ($\text{C}_{81}\text{H}_{80}\text{NO}_{21}\text{P}$ requires M : 1433.5012; $\text{C}_{75}\text{H}_{64}\text{O}_{21}\text{P}^-$ requires m/z 1331.3683).

2,3,6-Tri-*O*-benzoyl-4-*O*-(2,3,4-tri-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl)- β -D-galactopyranosyl)- α -D-mannopyranose **35**



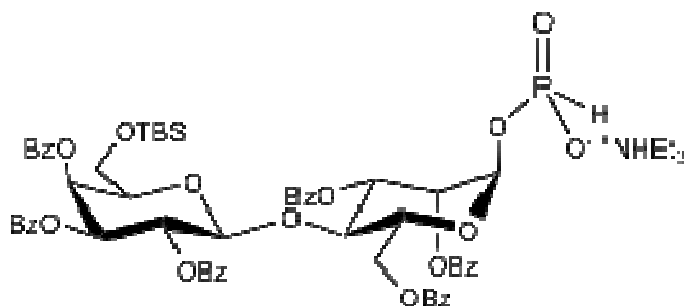
Acetic acid (37 μ l, 0.277 mmol) was added dropwise to a stirring solution of ethylenediamine (37 μ l, 0.555 mmol) in THF (2 ml). This instantly formed a white precipitate. The protected disaccharide **30** (300 mg, 0.277 mmol) was added and the reaction mixture was left for 20 h under stirring. TLC (Sovlent G) showed the reaction was complete. Water was added and, after 10 min, the reaction mixture was diluted with CH_2Cl_2 and washed successively with 1M HCl, water and saturated aq. NaHCO_3 , dried (MgSO_4) and concentrated. FCC [toluene-ethyl acetate (85:15)] of the residue yielded the hemiacetal derivative **35** (282 mg, 0.241 mmol, 87 %) as an amorphous solid.

^1H NMR (CDCl_3): δ_{H} -0.08 (3H, s, CH_3Si), 0.00 (3H, s, CH_3Si), 0.92 (9H, s, *tert*-Bu), 3.41-3.49 (2H, m, H-6b' & H-6a'), 3.73 (1H, ddd, $J_{5',6a'}$ 5.7, $J_{5',6b'}$ 8.8, H-5'), 4.58 (1H, ddd, $J_{5,6a}$ 2.9, H-5), 4.67 (1H, dd, $J_{6a,6b}$ 12.2, H-6a), 4.75 (1H, t, $J_{3,4} = J_{4,5} = 9.6$, H-4), 4.96 (1H, dd, $J_{5,6b}$ 1.9, H-6b), 5.18 (1H, d, $J_{1',2'}$ 7.9 H-1'), 5.57 (1H, br, H-1) 5.69 (1H, dd, $J_{2',3'}$ 10.5, $J_{3',4'}$ 3.4, H-3'), 5.88-5.93 (2H, m, H-2 & H-2'), 5.96 (1H, dd, $J_{4',5'}$ 0.8, H-4'), 6.22 (1H, dd, $J_{2,3}$ 3.4, H-3) and 7.36-8.25 (30H, m, Ph).

^{13}C NMR (CDCl_3): δ_{C} -5.94, -5.79 (CH_3Si), 18.01 (Me_3C), 25.63, (Me_3C), 59.35 (C-6'), 62.21 (C-6), 67.06 (C-4'), 69.34 (C-5), 70.46 (C-2' & C-3), 70.73 (C-2), 71.86 (C-3'), 73.45 (C-4), 73.82 (C-5'), 92.22 (C-1), 101.49 (C-1'), 128.19-133.31 (Ph) and 164.06-166.67 (C=O).

High resolution ES-MS(+): found m/z 1103.3498 $[\text{M} + \text{Na}]^+$ ($\text{C}_{60}\text{H}_{60}\text{O}_{17}\text{Si}$ requires M: 1080.3600; $\text{C}_{60}\text{H}_{60}\text{NaO}_{17}\text{Si}$ requires m/z 1103.3492).

2,3,6-Tri-*O*-benzoyl-4-*O*-(2,3,4-tri-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl- β -D-galactopyranosyl)- α -D-mannopyranosyl hydrogenphosphonate, triethylammonium salt **36**



Compound **35** (70 mg, 0.065 mmol) was dissolved in MeCN (2 ml), and to this solution triethylamine (600 μl , 4.24 mmol) and salicyl chlorophosphite (89 mg, 0.437 mmol) were added under stirring. Monitoring by TLC (Solvent H) showed complete conversion of **35** after 1 h. The reaction mixture was diluted with CHCl_3 , and washed successively by saturated aq. NaHCO_3 , water and cold 0.5M TEAB buffer solution,

dried by filtration through cotton wool and concentrated. FFC [CH_2Cl_2 -methanol (9:1)] of the residue gave the hydrogenphosphonate derivative **36** (79 mg, 0.0634 mmol, 98 %; a pure α -anomer at D-mannose residue) as an amorphous solid.

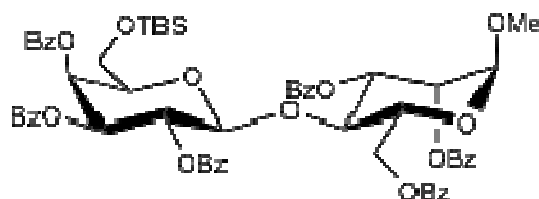
^1H NMR (CDCl_3): δ_{H} -0.07 (3H, s, CH_3Si), 0.00 (3H, s, CH_3Si), 0.92 (9H, s, *tert*-Bu), 1.58 (9H, t, 3 x MeCH_2), 3.28 – 3.34 (6H, m, 3 x MeCH_2), 3.41-3.49 (2H, m, H-6a' & 6b'), 3.74 (1H, dd, $J_{5',6a'}$ 5.9, $J_{5',6b'}$ 8.1, H-5'), 4.61 (1H, br d, H-5), 4.72 (1H, t, $J_{3,4} = J_{4,5} = 9.5$, H-4), 4.73 (1H, m, H-6a), 4.84 (1H, br d, $J_{6a,6b}$ 12.2, H-6b), 5.15 (1H, d, $J_{1',2'}$ 7.9, H-1'), 5.66 (1H, dd, $J_{3',4'}$ 3.3, H-3'), 5.87 (1H, dd, $J_{2',3'}$ 10.3, H-2'), 5.92-5.98 (3H, m, H-1, H-2 & H-4'), 6.14 (1H, dd, $J_{2,3}$ 3.2, H-3), 7.25 (1H, d, $J_{\text{H,p}}$ 638.4, HP) and 7.30-8.30 (30H, m, Ph).

^{13}C NMR (CDCl_3): δ_{C} -5.80 (CH_3Si), 8.57 (MeCH_2), 45.68 (MeCH_2), 18.01 (Me_3C), 25.63, (Me_3C), 59.45 (C-6'), 62.53 (C-6), 67.14 (C-4'), 70.24 (C-5), 70.37 (C-2'), 70.71 (C-3), 70.91 (br, C-2), 71.94 (C-3'), 73.41 (C-4), 73.51 (C-5'), 92.85 (br, C-1), 101.30 (C-1'), 128.12-133.15 (Ph) and 164.06-166.67 (C=O).

^{31}P NMR (CDCl_3): δ_{P} 0.50.

ES-MS(+): found m/z 1246.4578 [$\text{M} + \text{H}]^+$ ($\text{C}_{66}\text{H}_{76}\text{NO}_{19}\text{PSi}$ requires M: 1245.4518; $\text{C}_{66}\text{H}_{77}\text{NO}_{19}\text{PSi}^+$ requires m/z 1246.4572).

Methyl 2,3,6-tri-*O*-benzoyl-4-*O*-(2,3,4-tri-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl- β -D-galactopyranosyl)- α -D-mannopyranoside **37**



A mixture of compounds **24** (750 mg, 0.999 mmol) and **25** (496 mg, 0.832 mmol g) was dissolved in freshly distilled CH_2Cl_2 (4 ml), before freshly activated molecular sieves 4Å (500 mg) were added. This suspension was cooled to $-40\text{ }^\circ\text{C}$ under stirring and trimethylsilyl trifluoromethanesulfonate (18 μl , 0.1 mmol) was injected through the septa. Monitoring by TLC (Solvent A) showed that after 90 minutes the glycosyl donor **24** was consumed. The reaction was quenched by the addition of triethylamine (500 μl) and left for 10 min. The mixture was diluted with CH_2Cl_2 , filtered through a Celite pad to remove solids and the filtrate was concentrated. FCC [toluene-ethyl acetate (100:0 \rightarrow 95:5)] of the residue gave the protected disaccharide **37** (676 mg, 0.570 mmol, 68 %) as an amorphous solid.

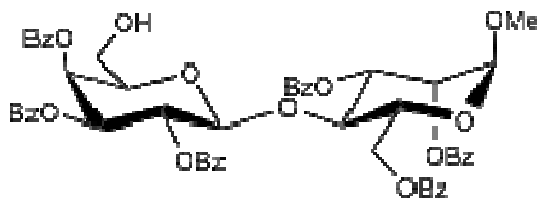
$[\alpha]_D^{20} +25.3$ (c 1, CHCl_3).

^1H NMR (CDCl_3): δ_{H} -0.09 (3H, s, CH_3Si), 0.00 (3H, s, CH_3Si), 0.92 (9H, s, *tert*-Bu), 3.43 (2H, d, $J_{5',6'}$ 7.2, H-6a' & H-6b'), 3.66 (3H, s, OMe), 3.73 (1H, br t, H-5'), 4.34 (1H, ddd, $J_{5,6a}$ 3.4, H-5), 4.69 (1H, dd, $J_{6a,6b}$ 11.9, H-6a), 4.72 (1H, t, $J_{3,4} = J_{4,5} = 9.6$, H-4), 4.92 (1H, dd, $J_{5,6b}$ 1.6, H-6b), 5.07 (1H, d, $J_{1,2}$ 1.8 H-1), 5.16 (1H, d, $J_{1',2'}$ 7.9, H-1'), 5.68 (1H,

dd, $J_{2',3'}$ 10.4, H-3'), 5.86 (1H, dd, $J_{2,3}$ 3.4, H-2), 5.90 (1H, dd, H-2'), 5.96 (1H, br d, $J_{3',4'}$ 3.3, H-4'), 5.79 (1H, dd, H-3) and 7.35-8.31 (30H, m, Ph).

^{13}C NMR (CDCl_3): δ_{c} -5.94, -5.80 (CH_3Si), 18.01 (Me_3C), 25.81 (Me_3C), 55.39 (OMe), 59.29 (C-6'), 62.33 (C-6), 67.00 (C-4'), 69.17 (C-5), 70.33 (C-2), 70.41 (C-2'), 70.73 (C-3), 71.89 (C-3'), 73.42 (C-5'), 73.88 (C-4), 98.49 (C-1), 101.47 (C-1'), 128.34–133.51 (Ph) and 164.06-166.67 (C=O).

Methyl 2,3,6-tri-*O*-benzoyl-4-*O*-(2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl)- α -D-mannopyranoside **38**



Acetonitrile (1 ml) was used to dissolve the disaccharide **37** (100 mg, 0.091 mmol), and the solution was cooled to 0 °C. A mixture of 40 % aq. HF and MeCN (1:1, 1 ml) was added under stirring. The reaction was run for 1 h and monitored by TLC (Solvent E). To stop the reaction saturated aq. NaHCO_3 was added dropwise to the mixture until the bubbling stopped. The reaction mixture was diluted with CH_2Cl_2 , washed with water (5 x 40 ml), dried (MgSO_4) and concentrated. FCC [toluene-ethyl

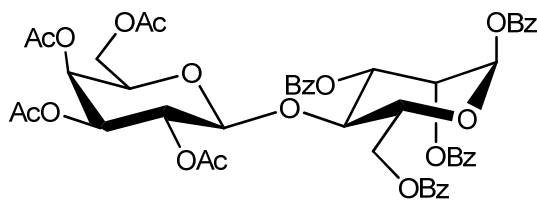
acetate (98:2→80:20)] of the residue yielded **38** (80 mg, 0.09 mmol, 98 %) as an amorphous solid.

^1H NMR (CDCl_3): δ_{H} 2.96 (1H, dd, $J_{6\text{a}',6\text{b}'}$ 12.1, H-6a'), 3.06 (1H, dd, $J_{5',6\text{b}'}$ 6.4, H-6b'), 3.36 (3H, s, OMe), 3.49 (1H, br dd, $J_{5',6\text{a}'}$ 7.1, H-5'), 4.05 (1H, ddd, $J_{5,6\text{a}}$ 3.5, H-5), 4.42 (1H, dd, $J_{6\text{a},6\text{b}}$ 12.2, H-6a), 4.45 (1H, t, $J_{3,4} = J_{4,5} = 9.6$, H-4), 4.62 (1H, dd, $J_{5,6\text{b}}$ 1.9, H-6b), 4.79 (1H, d, $J_{1,2}$ 1.9 H-1), 4.87 (1H, d, $J_{1',2'}$ 7.9, H-1'), 5.33 (1H, dd, $J_{2',3'}$ 10.4, H-3'), 5.51 (1H, br d, $J_{3',4'}$ 3.4, H-4'), 5.53 (1H, dd, $J_{2,3}$ 3.4, H-2), 5.71 (1H, dd, H-2'), 5.79 (1H, dd, H-3) and 7.06-8.11 (30H, m, Ph).

^{13}C NMR (CDCl_3): δ_{C} 55.42 (OMe), 59.90 (C-6'), 62.42 (C-6), 68.51 (C-4'), 69.20 (C-5), 70.21 (C-2 & C-2'), 70.59 (C-3), 71.77 (C-3'), 73.82 (C-5'), 74.00 (C-4), 98.49 (C-1), 101.21 (C-1'), 128.34-133.51 (Ph) and 164.06-166.67 (C=O).

High resolution ES-MS(+): found m/z 1003.2761 $[\text{M} + \text{Na}]^+$ ($\text{C}_{55}\text{H}_{48}\text{O}_{17}$ requires M: 980.2892; $\text{C}_{55}\text{H}_{48}\text{NaO}_{17}^+$ requires m/z 1003.2784).

1,2,3,6-Tetra-*O*-benzoyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl- β -D-galactopyranosyl)- α -D-mannopyranose **39**



A mixture of compounds **29** (300 mg, 0.609 mmol) and **21** (330 mg, 0.553 mmol) was dissolved in freshly distilled CH_2Cl_2 (5 ml), before freshly activated molecular sieves 4\AA (500 mg) were added. This suspension was cooled to $-40\text{ }^\circ\text{C}$ under stirring and trimethylsilyl trifluoromethanesulfonate (39 μl , 0.216 mmol) was injected through the septa. Monitoring by TLC (Solvent D) showed that after 30 minutes the glycosyl donor **29** was consumed. The reaction was quenched by the addition of a mixture of methanol (10 μl), triethylamine (10 μl) and CH_2Cl_2 (1 ml) and left for 10 min. The mixture was diluted with CH_2Cl_2 , filtered through a Celite pad to remove solids and the filtrate was concentrated. FCC [toluene- ethyl acetate (100:0 \rightarrow 95:5)] of the residue gave the disaccharide **39** (496 mg, 0.536 mmol, 88 %) as an amorphous solid.

$$[\alpha]_D^{20} +14.1 \text{ (c 1, CHCl}_3\text{)}.$$

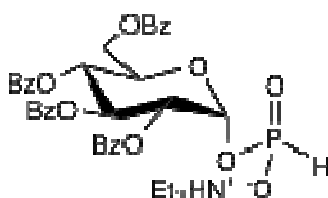
^1H NMR (CDCl_3): δ_{H} 1.69, 1.85, 1.92, 1.94 (12H, 4 x s, 4 x Ac), 3.37 (1H, br dd, $J_{5',6a'}$ 5.8, H-5'), 3.42 (1H, dd, $J_{6a',6b'}$ 10.8, H-6'), 3.67 (1H, dd, $J_{5',6b'}$ 7.8, H-6b), 4.25 (1H, ddd, $J_{5,6a}$ 3.3, H-5), 4.44 (1H, dd, $J_{6a,6b}$ 12.2, H-6a), 4.53 (1H, t, $J_{3,4} = J_{4,5} = 9.6$, H-4), 4.65 (1H, d, $J_{1',2'}$ 8.0, H-1'), 4.71 (1H, dd, $J_{5,6b}$ 2.0, H-6b), 4.80 (1H, dd, $J_{3',4'}$ 3.4, H-3'), 5.09 (2H, m,

H-2' & H-4'), 5.78 (1H, dd, $J_{2,3}$ 3.4, H-2), 5.87 (1H, dd, H-3), 6.45 (1H, d, $J_{1,2}$ 2.1, H-1) and 7.05-8.11 (20H, m, Ph).

^{13}C NMR (CDCl_3): δ_{c} 20.32, 20.53, 20.70 (MeCO), 60.28 (C-6'), 62.18 (C-6), 66.37 (C-4'), 69.26 (C-2'), 69.32 (C-2), 70.33 (C-3), 70.65 (C-5'), 70.96 (C-3'), 71.54 (C-5), 73.45 (C-4), 91.27 (C-1), 101.20 (C-1'), 128.25-134.11 (Ph), 164.02, 164.94, 165.84 (PhCO_2) and 169.36, 169.88, 170.10 (MeCO_2).

High resolution ES-MS(+): found m/z 949.2572 $[\text{M} + \text{Na}]^+$ ($\text{C}_{48}\text{H}_{46}\text{O}_{19}$ requires M: 926.2633; $\text{C}_{48}\text{H}_{46}\text{NaO}_{19}^+$ requires m/z 949.2526).

2,3,4,6-Tetra-*O*-benzoyl- α -D-glucopyranosyl hydrogenphosphonate, triethylammonium salt 40



To a stirred solution of imidazole (486 mg, 7.20 mmol) in MeCN (12 ml) at 0 °C was added phosphorus trichloride (189 μl , 2.16 mmol) and then triethylamine (1.05 ml, 7.54 mmol). After 15 min, a solution of compound **53** (2,3,4,6-tetra-*O*-benzoyl- α -D-glucopyranose) (300 mg, 0.5 mmol) in MeCN (7 ml) was added dropwise during 15 mins at 0 °C. After 30 min at rt, the reaction was quenched with cold 1M TEAB

buffer solution (3 ml). After a further 15 min, the mixture was diluted with CH₂Cl₂ (40 ml) and washed successively with ice-water (twice) and cold 0.5M TEAB solution (twice), dried by filtration through cotton wool and concentrated. FFC [CH₂Cl₂-methanol (95:5)] of the residue gave the H-phosphonate derivative **40** (282 mg, 0.39 mmol, 78 %) as an amorphous solid.

$[\alpha]_D^{20} +65.7$ (c 1, CHCl₃).

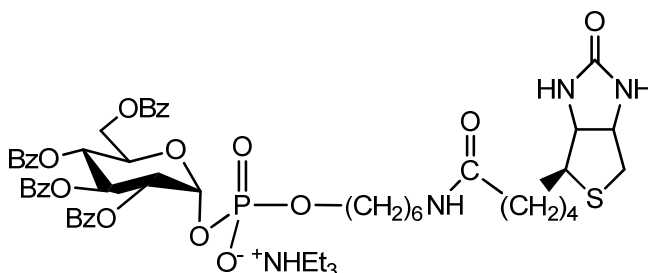
¹H NMR (CDCl₃): δ_H 1.10 (9H, t, 3 x MeCH₂), 3.27 (6H, m, 3 x MeCH₂), 4.36 (1H, dd, *J*_{6a,6b} 12.3, H-6a), 4.55 (1H, dd, *J*_{5,6b} 2.9, H-6b), 4.73 (1H, ddd, *J*_{5,6a} 4.3, H-5), 5.32 (1H, ddd, ⁴*J*_{2,P} 1.2, H-2), 5.69 (1H, t, *J*_{3,4} = *J*_{4,5} = 9.9, H-4), 6.01 (1H, dd, *J*_{1,2} 3.4, *J*_{1,P} 8.8, H-1), 6.22 (1H, t, *J*_{2,3} 9.9, H-3), 6.99 (1H, d, ¹*J*_{H,P} 638, HP) and 7.17-8.00 (20H, m, Ph).

¹³C NMR (CDCl₃): δ_C 8.55 (MeCH₂), 45.52 (MeCH₂), 62.78 (C-6), 68.71 (C-5), 69.37 (C-4), 70.48 (C-3), 71.83 (d, ³*J*_{C,P} 5.7, C-2), 91.60 (d, ²*J*_{C,P} 4.2, C-1), 128.26-133.82 (Ph) and 165.25-165.71 (C=O).

³¹P NMR (CDCl₃): δ_P 0.82.

High resolution ES-MS(-): found *m/z* 659.1315 [M - Et₃N - H]⁻ (C₄₀H₄₄NO₁₂P⁻ requires M, 761.2601, C₃₄H₂₈O₁₂P⁻ requires *m/z* 659.1324).

6-*N*-Biotinylaminoethyl 2,3,4,6-tetra-*O*-benzoyl- α -D-glucopyranosyl phosphate, triethylammonium salt **41**



A mixture of compounds **40** (200 mg, 0.26 mmol) and **27** (82 mg, 0.23 mmol) was dried by evaporation of pyridine (3 x 5 ml) therefrom. The residue was dissolved in pyridine (2 ml), pivaloyl chloride (96 μ l, 0.78 mmol) was added and the mixture was left to stir at rt for 90 min, whereafter TLC (Solvent A) showed formation of a new spot. A freshly prepared solution of iodine (230 mg, 0.91 mmol) in pyridine-water (95:5, 1 ml) was added. After 30 min, the mixture was diluted with CH_2Cl_2 and washed successively with ice-cold 1M aq. $\text{Na}_2\text{S}_2\text{O}_3$, cold 0.5M TEAB buffer and water, dried by filtration through cotton wool and concentrated. FCC [CH_2Cl_2 -methanol (100:0 \rightarrow 95:5)] of the residue gave the phosphodiester **41** (190 mg, 0.177 mmol, 77 %) as an amorphous solid.

^1H NMR (CDCl_3): δ_{H} 1.16 (9H, t, 3 x MeCH_2), 1.25-1.63 (14H, m, 7 x CH_2), 2.13 (2H, t, J 7.4, CH_2CO), 2.66 (1H, br d, $J_{\text{a,b}}$ 12.8, H^{b}), 2.87 (1H, dd, $J_{\text{a,c}}$ 4.9, H^{a}), 2.87 (6H, q, 3 x MeCH_2), 3.02-3.07 (1H, m, H^{e}), 3.09-3.17 (2H, q, J 6.1, CH_2N), 3.72-3.85 (2H, m, CH_2OP), 4.23 (1H, m, H^{d}), 4.33 (1H, dd, $J_{6\text{a},6\text{b}}$ 12.3, H-6a), 4.40 (1H, m, H^{c}), 4.63 (1H, dd, $J_{5,6\text{b}}$ 2.8, H-6b), 4.73 (1H, ddd, $J_{5,6\text{a}}$ 5.8, H-5), 5.10 (1H, br, NH), 5.24 (1H, ddd, $^4J_{2,\text{P}}$

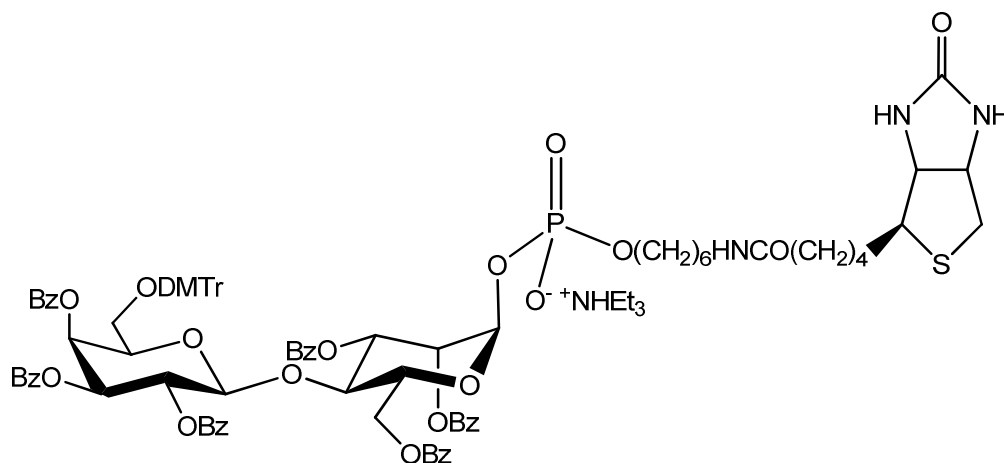
1.7, H-2), 5.74 (1H, t, $J_{3,4} = J_{4,5} = 9.9$, H-4), 5.96 (1H, dd, $J_{1,2} 3.3$, $J_{1,p} 7.8$, H-1), 6.05 (1H, br, NH), 6.22 (1H, t, $J_{2,3} 9.9$, H-3), 7.75 (1H, m, CH₂NH) and 7.18-8.02 (20H, m, Ph).

¹³C NMR (CDCl₃): δ_c 8.51 (MeCH₂), 24.86, 25.75, 25.98, 28.08, 29.16, 29.71 (6 x CH₂), 30.16 (d, $^3J_{C,p} 8.1$, CH₂CH₂OP), 35.80 (CH₂CO), 38.98 (CH₂N), 40.55 (C^{ab}), 45.70 (MeCH₂), 55.62 (C^e), 60.25 (C^c), 61.95 (C^d), 62.61 (C-6), 65.61 (br, CH₂OP) 68.50 (C-5), 69.20 (C-4), 70.42 (C-3), 72.11 (d, $^3J_{C,p} 8.0$, C-2), 92.24 (br, C-1), 128.32-133.42 (Ph), 165.23, 165.75, 166.24 (PhC=O) and 173.37 (CH₂CONH).

³¹P NMR (CDCl₃): δ_p -2.18.

High resolution ES-MS(-): found m/z 1000.3125 [M - Et₃N - H]⁻ (C₅₆H₇₁N₄O₁₅PS requires M: 1102.4374; C₅₀H₅₅N₃O₁₅SP⁻ requires m/z 1000.3097).

6-*N*-Biotinylaminohexyl 2,3,6-tri-*O*-benzoyl-4-*O*-[2,3,4-tri-*O*-benzoyl-6-*O*-(*p,p'*-dimethoxytrityl)- β -D-galactopyranosyl]- α -D-mannopyranosyl phosphate, triethylammonium salt **42**

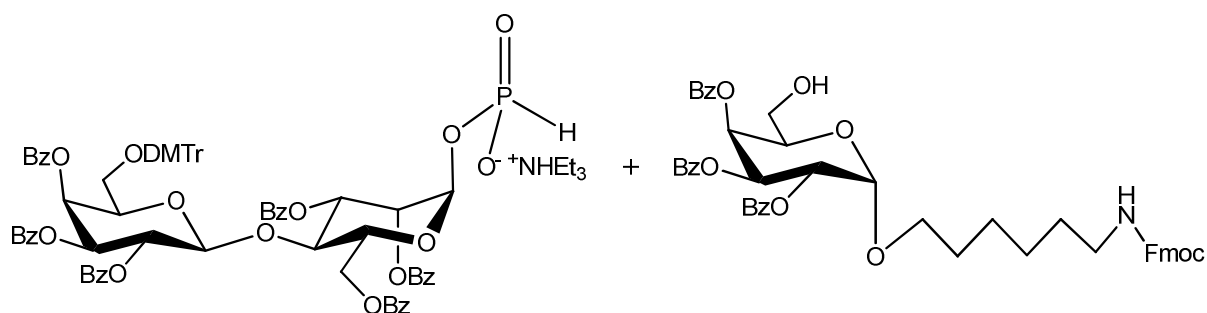


A mixture of compounds **34** (80 mg, 0.056 mmol) and **27** (20 mg, 0.056 mmol) was dried by evaporation of pyridine (3 x 5 ml) therefrom. The residue was dissolved in pyridine (1 ml), pivaloyl chloride (21 μ l, 0.167 mmol) was added and the mixture was left to stir at rt for 90 min, whereafter TLC (Solvent A) showed formation of a new spot. A freshly prepared solution of iodine (42 mg, 0.112 mmol) in pyridine-water (95:5, 1 ml) was added. After 30 min, the mixture was diluted with CH_2Cl_2 and washed successively with ice-cold 1M aq. $\text{Na}_2\text{S}_2\text{O}_3$, cold 0.5M TEAB buffer and water, dried by filtration through cotton wool and concentrated. TLC (Solvent H) showed a very faint spot, which was thought to be the product. FCC [CH_2Cl_2 -methanol (100:0 \rightarrow 95:5)] of the residue gave the biotin linked disaccharide phosphate **42** (8 mg, 0.0045 mmol, 8%) as an amorphous solid.

^1H NMR (CD_3OD): δ_{H} 1.15-1.50 (m, 3 x MeCH_2 & 7 x CH_2), 2.24 (m, CH_2CO), 2.90-3.25 (m, 3 x MeCH_2 , H-6a', H-6b', H^{a} , H^{b} , H^{e} & CH_2NH), 3.52 (m, CH_2OP), 3.56, 3.57, 3.58, 3.59 (4 x s, 2 x OMe, signals duplicated due to possible rotamers), 3.65 (m, H-5'), 4.05 (m, H^{d}), 4.23 (m, H-5 & H^{c}), 4.35-4.56 (m, H-4, H-6a & H-6b), 4.80, 4.84 (2 x d, $J_{1',2'}$ 7.9, H-1', signals duplicated due to possible rotamers), 5.38 (m, H-3'), 5.47-5.62 (m, H-1, H-2, & H-2'), 5.64, 5.72 (2 x dd, $J_{2,3}$ 3.2, $J_{3,4}$ 9.7, H-3, signals duplicated due to possible rotamers), 5.87 (d, $J_{3',4'}$ 3.3, H-4') and 6.45-7.92 (m, 7 x Ph & 2 x C_6H_4).

^{31}P NMR (CDCl_3): δ_{p} -2.18.

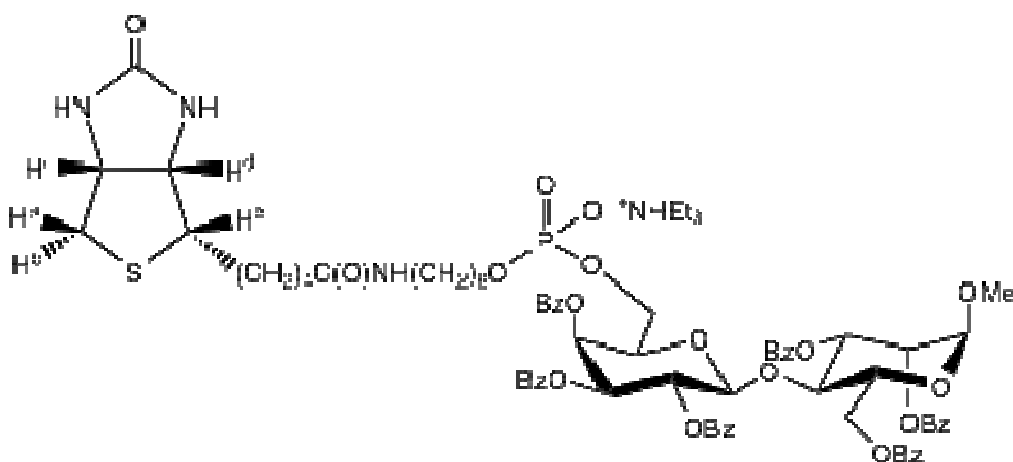
Attempted coupling of 2,3,6-tri-O-benzoyl-4-O-[2,3,4-tri-O-benzoyl-6-O-(p,p'-dimethoxytrityl)- β -D-galactopyranosyl]- α -D-mannopyranosyl hydrogenphosphonate **34 and the Gal-linker-Fmoc derivative **43****



A mixture of compounds **34** (80 mg, 0.056 mmol) and **43** (63 mg, 0.077 mmol) was dried by evaporation of pyridine (3 x 4 ml) therefrom. The residue was dissolved in

pyridine (1.5 ml), pivaloyl chloride (26 μ l, 0.21 mmol) was added and the mixture was left to stir at rt for 2 h, whereafter a freshly prepared solution of iodine (36 mg, 0.140 mmol) in pyridine-water (95:5, 1 ml) was added. After 30 min, the mixture was diluted with CH_2Cl_2 and washed successively with ice-cold 1M aq. $\text{Na}_2\text{S}_2\text{O}_3$, cold 0.5M TEAB buffer and water, dried by filtration through cotton wool and concentrated. It could be seen by TLC (Solvent H) that no expected phosphodiester product was formed.

Methyl 2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranoside 6-(6-*N*-biotinylaminohexyl phosphate), triethylammonium salt **44**



A mixture of the H-phosphonate **28** (101.4 mg, 0.249 mmol) and the disaccharide **38** (122 mg, 0.124 mmol) was dried by evaporation of pyridine (3 x 5 ml) therefrom. The residue was dissolved in pyridine (2 ml) and triethylamine (70 μ l, 0.498 mmol) was added. Pivaloyl chloride (107 μ l, 0.868 mmol) was then added and the mixture was left to stir at rt for 60 min, whereafter TLC (Solvent A) showed that the disaccharide **38** was consumed. A freshly prepared solution of iodine (126.3 mg, 0.498 mmol) in pyridine-water (95:5, 1 ml) was added. After 20 min, the mixture was diluted with CH_2Cl_2 and washed successively with ice-cold 1M aq. $\text{Na}_2\text{S}_2\text{O}_3$, cold 0.5M TEAB buffer and water, dried by filtration through cotton wool and concentrated. TLC (Solvent H) showed a strong new spot, which was thought to be the product.

FCC [CH₂Cl₂-methanol (90:10→80:20)] of the residue gave the protected biotin linked disaccharide phosphate **44** (165 mg, 0.104 mmol, 84%) as an amorphous solid.

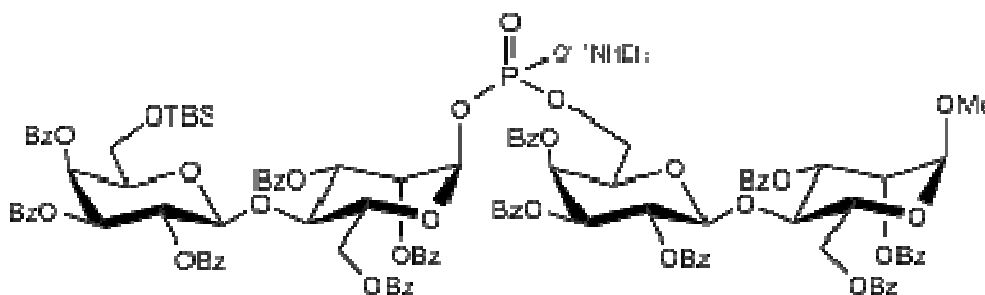
¹H NMR (CDCl₃): δ_H 1.16-1.72 (23H, m, 3 x MeCH₂ & 7 x CH₂), 2.10 (2H, m, CH₂CO), 2.64 (1H, br d, *J*_{a,b} 12.5, H^b), 2.92 (1H, dd, *J*_{a,c} 5.0, H^a), 2.94 (6H, q, 3 x MeCH₂), 3.01 (1H, m, H^e), 3.08 (2H, m, CH₂N), 3.31 (1H, m, H-6a'), 3.35 (3H, s, OMe), 3.49-3.62 (2H, m, CH₂OP), 3.78 (1H, m, H-6b'), 3.99 (1H, dt, *J*_{5,6} 2.9, H-5), 4.04 (1H, m, H-5'), 4.17 (1H, m, H^d), 4.33 (1H, m, H^c), 4.43 (1H, t, *J*_{3,4} = *J*_{4,5} = 9.7, H-4), 4.53 (2H, d, *J*_{5,6} 2.9, H-6a & H-6b), 4.79 (1H, d, *J*_{1,2} 1.5 H-1), 4.92 (1H, d, *J*_{1',2'} 7.9, H-1'), 5.34 (1H, dd, *J*_{3',4'} 3.3, H-3'), 5.53 (1H, dd, *J*_{2,3} 3.4, H-2), 5.58 (1H, dd, *J*_{2',3'} 10.3, H-2'), 5.74 (2H, m, H-3 & H-4'), 6.38 (1H, br, NH) 6.71 (1H, br, NH) and 7.06-8.00 (31H, m, CH₂NH & Ph).

¹³C NMR (CDCl₃): δ_C 8.50 (MeCH₂), 24.78, 25.77, 27.96, 28.88, 29.70 (6 x CH₂), 30.02 (br, CH₂CH₂OP), 35.80 (CH₂CO), 38.95 (CH₂N), 40.61 (C^{ab}), 45.45 (MeCH₂), 55.39 (OMe), 55.68 (C^e), 60.07 (C^c), 61.39 (br, C-6'), 62.00 (C^d), 62.76 (C-6), 64.97 (br, CH₂OP), 67.29 (C-4'), 69.61 (C-5), 69.70 (C-3), 70.18 (C-2'), 70.76 (C-2), 70.91 (br, C-5'), 72.49 (C-3'), 73.51 (C-4), 98.41 (C-1), 101.07 (C-1'), 128.25-133.21 (Ph), 165.25, 165.42, 165.60 (PhC=O) and 173.38 (CH₂CONH).

³¹P NMR (CDCl₃): δ_P -0.47.

High resolution ES-MS(+): found *m/z* 1386.4489 [M - Et₃N + H]⁺ (C₇₇H₉₁N₄O₂₂PS requires M: 1486.5583; C₇₁H₇₇N₃O₂₂PS⁺ requires *m/z* 1386.4452) and 1408.4313 [M - Et₃N + Na]⁺ (C₇₁H₇₆N₃NaO₂₂PS⁺ requires *m/z* 1408.4276).

Methyl 2,3,4-tri-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranoside 6-[2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranosyl phosphate] triethylammonium salt **45**



A mixture of compounds **36** (65 mg, 0.052 mmol) and **38** (42.7 mg, 0.044 mmol) was dried by evaporation of pyridine (3 x 5 ml) therefrom. The residue was dissolved in pyridine (2 ml), pivaloyl chloride (31 μ l, 0.25 mmol) was added and the mixture was left to stir at rt for 90 min, whereafter TLC (Solvent A) showed that the H-phosphonate **36** was consumed. A freshly prepared solution of iodine (32.1 mg, 0.125 mmol) in pyridine-water (95:5, 1 ml) was added. After 1 h, the mixture was diluted with CH₂Cl₂ and washed successively with ice-cold 1M aq. Na₂S₂O₃, cold 0.5M TEAB buffer and water, dried by filtration through cotton wool and concentrated. FCC [CH₂Cl₂-methanol (95:5 \rightarrow 90:10)] of the residue gave the protected tetrasaccharide phosphate **45** (80 mg, 0.036 mmol, 82 %) as a amorphous solid.

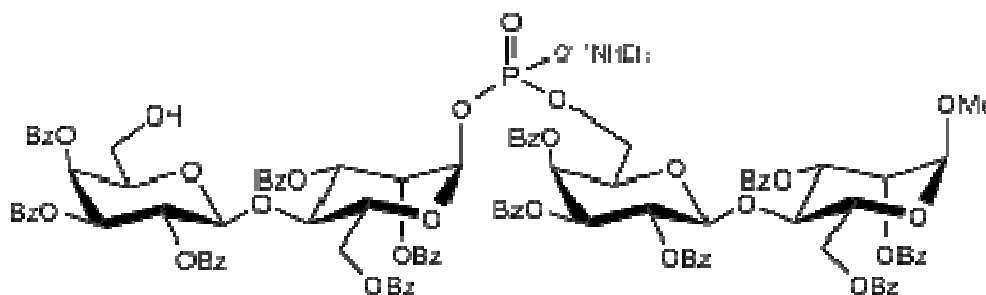
^1H NMR (CDCl_3): δ_{H} : -0.08 (3H, s, CH_3Si), 0.00 (3H, s, CH_3Si), 0.94 (9H, s, *tert*-Bu), 1.58 (9H, t, 3 x MeCH_2), 3.27 (6H, m, 3 x MeCH_2), 3.39-3.49 (3H, m, H-6a Gal, H-6a & H-6b Gal'), 3.67 (3H, s, OMe), 3.69 (1H, m, H-5 Gal'), 4.14-4.20 (1H, m, H-6b Gal), 4.29 (1H, ddd, $J_{4,5}$ 9.7, $J_{5,6a}$ 2.1, $J_{5,6b}$ 4.1, H-5 Man'), 4.40 (1H, dd, $J_{5,6a}$ 9.3, $J_{5,6b}$ 5.6, H-5 Gal), 4.48-4.88 (7H, m, H-4, H-5, H-6a H-6b Man & H-4, H-6a, H-6b Man'), 5.12 (1H, d, $J_{1,2}$ 1.8, H-1 Man), 5.13 (1H, d, $J_{1,2}$ 7.8, H-1 Gal'), 5.23 (1H, d, $J_{1,2}$ 7.8, H-1 Gal), 5.65 (2H, dd, $J_{2,3}$ 10.4, $J_{3,4}$ 3.3, H-3 Gal & H-3 Gal'), 5.71 (1H, dd, $J_{1,2}$ 1.9, $J_{1,p}$ 7.0, H-1 Man'), 5.81 (1H, dd, $J_{2,3}$ 3.5, H-2 Man), 5.86-5.97 (4H, m, H-2 Gal, H-2 Man' & H-2, H-4 Gal'), 6.07-6.12 (3H, m, H-3 Man, H-3 Man' & H-4 Gal) and 7.35-8.32 (60H, m, Ph).

^{13}C NMR (CDCl_3): δ_{C} -5.94, -5.82 (CH_3Si), 8.65 (MeCH_2), 17.86 (Me_3C), 25.61 (Me_3C), 45.71 (MeCH_2), 55.35 (OMe), 59.35 (C-6 Gal'), 61.46 (br, C-6 Gal), 62.17 (C-6 Man), 62.49 (C-6 Man'), 67.03 (C-4 Gal'), 67.15 (C-4 Gal), 69.23 (C-3 Man), 69.78 (C-5 Man'), 69.86 (C-5 Man'), 70.39 (C-2 Gal), 70.43 (C-2 Gal'), 70.52 (d, $J_{\text{C,p}}$ 7.5, C-2 Man'), 70.62 (C-3 Man'), 71.02 (C-2 Man), 72.07 (d, $J_{\text{C,p}}$ 5.6, C-5 Gal & C-3 Gal'), 72.58 (C-3 Gal), 73.16 (C-4 Man'), 73.32 (C-5 Gal'), 74.03 (C-4 Man), 93.57 (br, C-1 Man') 98.41 (C-1 Man), 101.15 (C-1 Gal'), 101.36 (C-1 Gal), 127.98-133.45 (Ph) and 164.32-165.55 (C=O).

^{31}P NMR (CDCl_3): δ_{P} -4.34.

High resolution ES-MS(-): found 2121.5985 m/z [$\text{M} - \text{Et}_3\text{N} - \text{H}$] $^-$ ($\text{C}_{121}\text{H}_{122}\text{NO}_{36}\text{PSi}$ requires M: 2223.7253; $\text{C}_{115}\text{H}_{106}\text{O}_{36}\text{PSi}^-$ requires m/z 2121.5976).

Methyl 2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranoside 6-[2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranosyl phosphate] triethylammonium salt
46



Compound **45** (250 mg, 0.112 mmol) was dissolved in MeCN (3 ml), and the solution was cooled to 0°C. A mixture of 40 % aq. HF and MeCN (1:1, 3 ml) was added under stirring. The reaction was run for 2 h, whereafter TLC (Solvent H) showed the deprotection was complete. Saturated aq. NaHCO₃ was added dropwise to the reaction mixture until the bubbling stopped. The mixture was then diluted with CHCl₃, washed successively with saturated aq. NaHCO₃, water, cold 0.5M TEAB buffer and water, dried by filtration through cotton wool and concentrated. FCC [CH₂Cl₂–methanol (100:0 \rightarrow 98:2)] gave the tetrasaccharide phosphate **46** (212.7 mg, 0.101 mmol, 90%) as a amorphous solid.

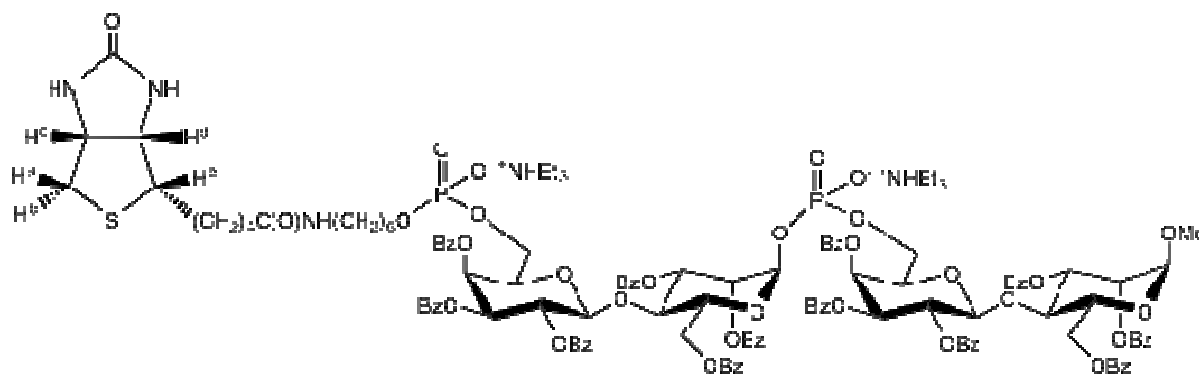
¹H NMR (CDCl₃): δ_{H} 1.17 (9H, t, 3 x MeCH₂), 2.91 (6H, m, 3 x MeCH₂), 2.97 (1H, dd, $J_{6a,6b}$ 12.0 H-6a Gal'), 3.10 (1H, dd, H-6b Gal'), 3.16 (1H, m, H-6a Gal), 3.34 (3H, s, OMe), 3.45 (1H, t, $J_{5,6a} = J_{5,6b} = 6.5$, H-5 Gal'), 3.85 (1H, m, H-6b Gal), 3.96 (1H, ddd,

$J_{4,5}$ 10.5, $J_{5,6a}$ 1.8, $J_{5,6b}$ 4.0, H-5 Man'), 4.08 (1H, dd, $J_{5,6a}$ 9.4, $J_{5,6b}$ 5.5, H-5 Gal), 4.26-4.56 (7H, m, H-4, H-5, H-6a, H-6b Man & H-4, H-6a, H-6b Man'), 4.79 (1H, d, $J_{1,2}$ 1.6, H-1 Man), 4.82 (1H, d, $J_{1,2}$ 7.9, H-1 Gal'), 4.91 (1H, d, $J_{1,2}$ 7.9, H-1 Gal), 5.27 (1H, dd, $J_{3,4}$ 3.4, H-3 Gal'), 5.33 (1H, dd, $J_{2,3}$ 10.4, H-3 Gal), 5.39 (1H, dd, $J_{1,2}$ 1.8, $J_{1,p}$ 6.8, H-1 Man'), 5.48 (2H, m, H-2 Man' & H-4 Gal'), 5.61 (2H, m, H-2 Man & H-2 Gal), 5.67 (1H, dd, $J_{2,3}$ 10.4, H-2 Gal'), 5.75 (1H, dd, $J_{2,3}$ 3.5, $J_{3,4}$ 9.7, H-3 Man'), 5.77 (1H, d, $J_{3,4}$ 3.3, H-4 Gal), 5.83 (1H, dd, $J_{2,3}$ 3.4, $J_{3,4}$ 9.6, H-3 Man) and 7.05-7.96 (60H, m, Ph).

^{13}C NMR (CDCl_3): δ_{c} 8.73 (MeCH_2), 45.41 (MeCH_2), 55.37 (OMe), 60.12 (C-6 Gal'), 61.56 (d, $J_{\text{C,p}}$ 7.1, C-6 Gal), 62.33 (C-6 Man & C-6 Man'), 67.18 (C-4 Gal), 68.51 (C-4 Gal'), 69.26 (C-3 Man'), 69.67 (C-5 Man & C-5 Man'), 69.87 (C-3 Man), 70.23 (C-2 Gal), 70.34 (C-2 Gal'), 70.80 (br, C-2 Man'), 70.94 (C-2 Man), 71.96 (br, C-5 Gal & C-3 Gal'), 72.53 (C-3 Gal), 73.73 (C-4 Man'), 74.00 (C-5 Gal'), 74.15 (C-4 Man), 93.51 (br, C-1 Man') 98.40 (C-1 Man), 101.16 (C-1 Gal'), 101.37 (C-1 Gal), 128.29-133.27 (Ph) and 164.93-166.45 (C=O).

^{31}P NMR (CDCl_3): δ_{p} -4.35.

Methyl 2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranoside 6-[2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranosyl phosphate 6-(6-*N*-biotinylaminohexyl phosphate)], bistriethylammonium salt **47**



A mixture of the H-phosphonate **28** (46.2 mg, 0.113 mmol) and the tetrasaccharide phosphate **46** (192 mg, 0.091 mmol) was dried by evaporation of pyridine (3 x 3 ml) therefrom. The residue was dissolved in pyridine (1 ml), the solution was cooled to 0 °C and triethylamine (31.9 μ l, 0.227 mmol) was added. Pivaloyl chloride (47.1 μ l, 0.399 mmol) was then added and the mixture was left to stir at rt for 60 min, whereafter a freshly prepared solution of iodine (57 mg, 0.227 mmol) in pyridine-water (95:5, 1 ml) was added. After 40 min, the mixture was diluted with CH₂Cl₂ and washed successively with ice-cold 1M aq. Na₂S₂O₃, cold 0.5M TEAB buffer and water, dried by filtration through cotton wool and concentrated. FCC [CH₂Cl₂-methanol (90:10 \rightarrow 80:20)] of the residue gave the protected biotin linked tetrasaccharide diphosphate **47** (188 mg, 0.072 mmol, 79 %) as an amorphous solid.

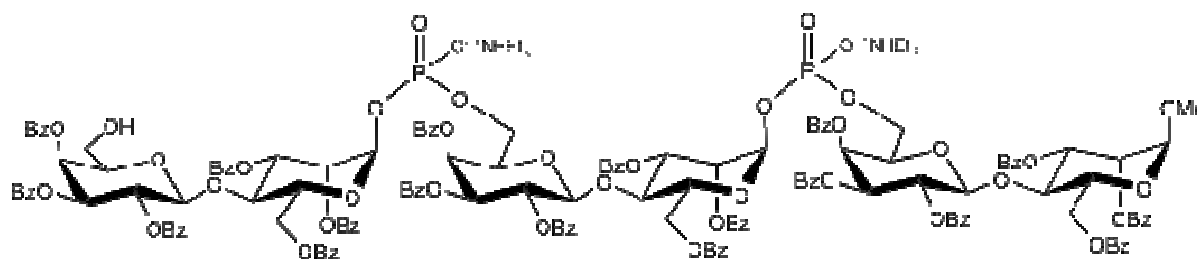
^1H NMR (CDCl_3): δ_{H} 1.11-1.70 (32H, m, 7 x CH_2 & 6 x MeCH_2), 2.05-2.11 (2H, m, CH_2CO), 2.61 (1H, d, $J_{\text{a,b}}$ 12.7, H^{b}), 2.77 (1H, dd, $J_{\text{a,c}}$ 5.0, H^{a}), 2.91 (12H, m, 6 x MeCH_2), 2.99-3.10 (3H, m, H^{e} & CH_2N), 3.14-3.25 (1H, m, H-6a Gal), 3.34 (3H, s, OMe), 3.35-3.44 (1H, m, H-6a Gal'), 3.47-3.60 (2H, m, CH_2OP), 3.74-3.80 (1H, m, H-6b Gal'), 3.84-3.91 (2H, m, H-6b Gal & H-5 Gal'), 3.97 (1H, dt, $J_{4,5}$ 9.5, $J_{5,6}$ 2.9, H-5 Man'), 4.09 (1H, dd, $J_{5,6\text{a}}$ 9.0, $J_{5,6\text{b}}$ 5.6, H-5 Gal), 4.14-4.19 (1H, m, H-5 Man & H^{d}), 4.29-4.40 (3H, m, H-6a, H-6b Man & H^{c}), 4.42-4.48 (2H, m, H-4 Man & H-4 Man'), 4.50-4.56 (2H, m, H-6a & H-6b Man'), 4.79 (1H, d, $J_{1,2}$ 1.6, H-1 Man), 4.89 (1H, d, $J_{1,2}$ 7.9, H-1 Gal'), 4.94 (1H, d, $J_{1,2}$ 7.9, H-1 Gal), 5.29 (1H, dd, $J_{2,3}$ 10.3, H-3 Gal'), 5.34 (1H, dd, $J_{2,3}$ 10.4, H-3 Gal), 5.39 (1H, dd, $J_{1,2}$ 1.7, $J_{1,\text{P}}$ 7.1, H-1 Man'), 5.49 (1H, dd, $J_{2,3}$ 3.4, H-2 Man), 5.55-5.63 (3H, m, H-2 Gal, H-2 Man' & H-2 Gal'), 5.71 (1H, d, $J_{3,4}$ 3.2, H-4 Gal'), 5.74 (1H, dd, $J_{2,3}$ 3.4, $J_{3,4}$ 10.3, H-3 Man'), 5.76 (1H, dd, $J_{3,4}$ 9.8, H-3 Man), 5.77 (1H, d, $J_{3,4}$ 3.3, H-4 Gal), 5.88 (1H, br, NH) 6.43 (1H, br, NH) and 7.08-7.99 (61H, m, CH_2NH & Ph).

^{13}C NMR (CDCl_3): δ_{C} 8.56 (MeCH_2), 24.83, 25.66, 25.75, 28.02, 28.93, 29.36 (6 x CH_2), 30.10 (br, $\text{CH}_2\text{CH}_2\text{OP}$), 35.82 (CH_2CO), 38.89 (CH_2N), 40.56 (C^{ab}), 45.51 (MeCH_2), 55.33 (OMe), 55.45 (C^{e}), 59.99 (C^{c}), 61.36 (br, C-6 Gal'), 61.56 (br, C-6 Gal), 61.94 (C^{d}), 62.35 (C-6 Man), 62.55 (C-6 Man'), 67.25 (C-4 Gal), 67.31 (C-4 Gal'), 69.32 (C-3 Man), 69.78 (C-3 Man'), 70.03 (C-5 Man'), 70.18 (C-5 Man & C-2 Gal'), 70.45 (C-2 Gal), 70.74 (d, $J_{\text{C,P}}$ 6.5, C-2 Man'), 70.99 (C-2 Man), 72.02 (br, C-5 Gal & C-5 Gal'), 72.52 (C-3 Gal & C-3 Gal'), 72.91 (C-4 Man'), 73.97 (C-4 Man), 93.52 (br, C-1 Man'), 98.40 (C-1 Man), 100.96 (C-1 Gal'), 101.29 (C-1 Gal), 127.98-133.48 (Ph), 164.85-166.20 (PhC=O) and 173.18 (CH_2CONH).

^{31}P NMR (CDCl_3): δ_{p} -4.43 (P), -0.64 (P').

High resolution ES-MS(-): found 1206.2823 m/z $[\text{M} - 2\text{Et}_3\text{N} - 2\text{H}]^{2-}$ ($\text{C}_{137}\text{H}_{151}\text{N}_5\text{O}_{41}\text{P}_2\text{S}$ requires M: 2615.9080; $\text{C}_{125}\text{H}_{119}\text{N}_3\text{O}_{41}\text{P}_2\text{S}^{2-}$ requires m/z 1205.8263) and 2412.5840 m/z $[\text{M} - 2\text{Et}_3\text{N} - \text{H}]^-$ ($\text{C}_{125}\text{H}_{120}\text{N}_3\text{O}_{41}\text{P}_2\text{S}^-$ requires m/z 2412.6599).

Methyl 2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranoside 6-{2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranosyl phosphate 6-[2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranosyl phosphate]}, bistriethylammonium salt **48**



A mixture of the H-phosphonate **34** (53 mg, 0.037 mmol) and the tetrasaccharide phosphate **46** (65 mg, 0.0308 mmol) was dried by evaporation of pyridine (3 x 3 ml) therefrom. The residue was dissolved in pyridine (1 ml), pivaloyl chloride (16.4 μl , 0.087 mmol) was added and the mixture was left to stir at rt for 2 h, whereafter a

freshly prepared solution of iodine (19 mg, 0.074 mmol) in pyridine-water (95:5, 1 ml) was added. After 3 h, the mixture was diluted with CHCl_3 and washed successively with ice-cold 1M aq. $\text{Na}_2\text{S}_2\text{O}_3$, cold 0.5M TEAB buffer and water, dried by filtration through cotton wool and concentrated. The residue was then dissolved in CH_2Cl_2 (5 ml) and 1 % TFA in CH_2Cl_2 (5 ml) was added at 0 °C. After 2 min, the solution was washed successively with cold saturated aq. NaHCO_3 and water, dried (MgSO_4) and concentrated. FFC [CH_2Cl_2 -methanol (90:10→85:15)] of the residue gave the hexasaccharide diphosphate **48** (68 mg, 0.019 mmol, 62%) as an amorphous solid.

^1H NMR (CDCl_3): δ_{H} 1.11 (18H, t, 6 x MeCH_2), 2.82 (12H, m, 3 x MeCH_2), 2.95 (1H, dd, $J_{5,6a}$ 6.4, $J_{6a,6b}$ 11.7 H-6a Gal''), 3.05-3.22 (3H, m, H-6a Gal, H-6a Gal' & H-6b Gal''), 3.33 (3H, s, OMe), 3.43 (1H, m, H-5 Gal''), 3.80 (1H, m, H-6b Gal'), 3.86 (1H, m, H-6b Gal), 3.90-3.98 (2H, m, H-5 Gal' & H-5 Man''), 4.06 (1H, dd, $J_{5,6a}$ 9.4, $J_{5,6b}$ 5.0, H-5 Gal), 4.14 (1H, br d, $J_{4,5}$ 9.4, H-5 Man'), 4.22-4.57 (10H, m, H-4, H-5, H6a, H6b Man, H-4, H-6a, H-6b Man' & H-4, H-6a H-6b Man''), 4.79 (2H, m, H-1 Man & H-1 Gal'), 4.83 (1H, d, $J_{1,2}$ 7.8, H-1 Gal''), 4.90 (1H, d, $J_{1,2}$ 7.7, H-1 Gal), 5.26 (2H, m, $J_{2,3}$ 10.1, H-3 Gal' & H-3 Gal''), 5.32 (2H, m, H-1 Man' or Man'' & H-3 Gal), 5.38 (1H, br d, $J_{1,p}$ 6.0, H-1 Man' or Man''), 5.48 (2H, m, H-2 Man'' & H-4 Gal''), 5.53-5.69 (5H, m, H-2 Man, H-2 Gal, H-2 Man', H-2 Gal', H-2 Gal''), 5.70-5.83 (5H, m, H-3 Man, H-4 Gal, H-3 Man', H-4 Gal', H-3 Man'') and 7.03-7.96 (90H, m, Ph).

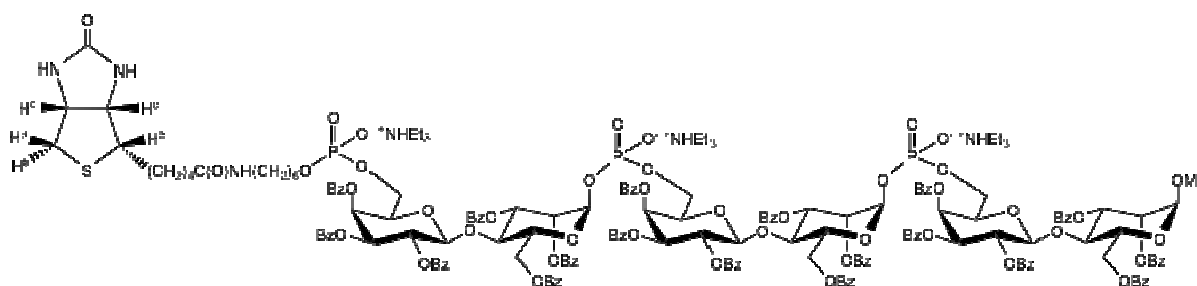
^{13}C NMR (CDCl_3): δ_{C} 9.65 (MeCH_2), 45.77 (MeCH_2), 55.36 (OMe), 60.05 (C-6 Gal''), 61.43k (2 x d, $J_{C,p}$ 4.4, $J_{C,p}$ 6.5, C-6 Gal & C-6 Gal'), 62.17, 62.38, 62.45 (C-6 Man, C-6

Man' & C-6 Man''), 67.09, 67.13 (C-4 Gal & C-4 Gal'), 68.47 (C-4 Gal''), 69.24 (C-3 Man), 69.50 (C-3 Man''), 69.76 (C-3 Man' & C-5 Man''), 69.84 (C-5 Man), 70.17 (C-5 Man'), 70.22 (C-2 Gal' & C-2 Gal''), 70.39 (C-2 Gal), 70.56 (d, $J_{C,P}$ 7.4, C-2 Man'), 70.81 (d, $J_{C,P}$ 7.2, C-2 Man''), 70.96 (C-2 Man), 71.83 (d, $J_{C,P}$ 7.4, C-5 Gal'), 71.89 (C-3 Gal''), 71.99 (d, $J_{C,P}$ 7.2, C-5 Gal), 72.51 (C-3 Gal & C-3 Gal'), 72.90 (C-4 Man'), 73.60 (C-4 Man''), 73.96 (C-4 Man), 74.12 (C-5 Gal''), 93.43 (br, C-1 Man' & C-1 Man''), 98.37 (C-1 Man), 100.46 (C-1 Gal'), 101.35 (C-1 Gal & C-1 Gal''), 128.12-133.71 (Ph) and 164.76-166.43 (C=O).

^{31}P NMR (CDCl_3): δ_p -4.47, -4.38 (P & P').

High resolution ES-MS(-): found m/z 1517.8813 $[\text{M} - 2\text{Et}_3\text{N} - 2\text{H}]^{2-}$ ($\text{C}_{175}\text{H}_{168}\text{N}_2\text{O}_{55}\text{P}_2$ requires M: 3238.9886; $\text{C}_{163}\text{H}_{136}\text{O}_{55}\text{P}_2^{2-}$ requires m/z 1517.3665) and 3035.8390 $[\text{M} - 2\text{Et}_3\text{N} - \text{H}]^-$ ($\text{C}_{163}\text{H}_{137}\text{O}_{55}\text{P}_2^-$ requires m/z 3035.7404).

Methyl 2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranoside 6-{2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranosyl phosphate 6-[2,3,4-tri-*O*-benzoyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2,3,6-tri-*O*-benzoyl- α -D-mannopyranosyl phosphate 6-(6-*N*-biotinylaminoethyl phosphate)]}, tris(ethylammonium) salt **49**



A mixture of the H-phosphonate **28** (11.3 mg, 0.0278 mmol) and the hexasaccharide diphosphate **48** (45 mg, 0.014 mmol) was dried by evaporation of pyridine (3 x 3 ml) therefrom. The residue was dissolved in pyridine (0.5 ml), the solution was cooled to 0 °C and triethylamine (7.75 μ l, 0.055 mmol) was added. Pivaloyl chloride (12.0 μ l, 0.097 mmol) was then added and the mixture was left to stir at rt for 2 h, whereafter a freshly prepared solution of iodine (14 mg, 0.055 mmol) in pyridine-water (95:5, 1 ml) was added. After 3 h, the mixture was diluted with CH₂Cl₂ and washed successively with ice-cold 1M aq. Na₂S₂O₃, cold 0.5M TEAB buffer and water, dried by filtration through cotton wool and concentrated. TLC (Solvent H) showed a strong spot; this was thought to be product. FCC [CH₂Cl₂–methanol (90:10 \rightarrow 80:20)] of the

residue gave the protected biotin linked hexasaccharide triphosphate **49** (35 mg, 0.009 mmol, 64 %) as an amorphous solid.

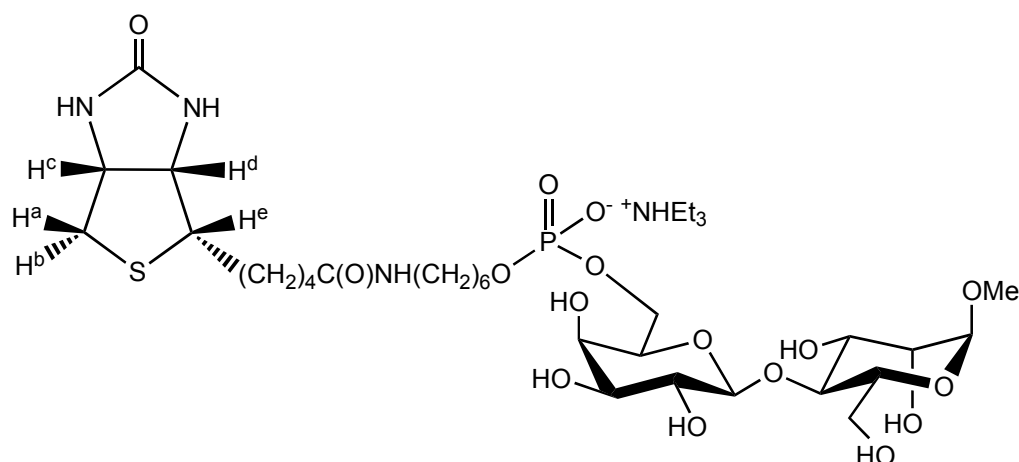
^1H NMR (CDCl_3): δ_{H} 1.10-1.72 (41H, m, 7 x CH_2 & 9 x MeCH_2), 2.02-2.10 (2H, m, CH_2CO), 2.64 (1H, d, $J_{\text{a,b}}$ 12.3, H^{b}), 2.75 (1H, dd, $J_{\text{a,c}}$ 4.9, H^{a}), 2.88 (18H, m, 9 x MeCH_2), 2.97-3.18 (5H, m, H-6a Gal, H-6a Gal', H^{e} & CH_2N), 3.33 (3H, s, OMe), 3.53-3.63 (3H, m, H-6a Gal'' & CH_2OP), 3.68-3.75 (1H, m, H-6b Gal'), 3.80-3.88 (3H, m, H-6b Gal, H-5 Gal' & H-5 Gal''), 3.91-4.03 (3H, m, H-5 Man', H-5 Man'' & H-6b Gal''), 4.07 (1H, dd, $J_{5,6\text{a}}$ 10.0, $J_{5,6\text{b}}$ 5.7, H-5 Gal), 4.12-4.56 (12H, m, H-4, H-5, H-6a, H-6b Man, H-4, H-6a, H-6b Man', H-4, H-6a, H-6b Man'', H^{c} & H^{d}), 4.79 (1H, br, H-1 Man), 4.91 (3H, d, $J_{1,2}$ 7.9, H-1 Gal, H-1 Gal' & H-1 Gal''), 5.26-5.41 (5H, m, H-3 Gal, H-1 Man', H-3 Gal', H-1 Man'' & H-3 Gal''), 5.47 (1H, m, H-2 Man), 5.53-5.63 (5H, m, H-2 Gal, H-2 Man', H-2 Gal', H-2 Man'' & H-2 Gal''), 5.66 (1H, m, H-4 Gal''), 5.72-5.79 (5H, m, H-3 Man, H-4 Gal, H-3 Man', H-4 Gal' & H-3 Man''), 6.53 (1H, br, NH), 7.03 (1H, br, NH) and 7.07-7.95 (91H, m, CH_2NH & Ph).

^{13}C NMR (CDCl_3): δ_{C} 8.56 (MeCH_2), 24.86, 25.72, 29.95, 27.52, 29.13, 29.50 (6 x CH_2), 30.23 (d, $J_{\text{C,P}}$ 9.3, $\text{CH}_2\text{CH}_2\text{OP}$), 35.72 (CH_2CO), 38.73 (CH_2N), 39.75 (C^{ab}), 45.74 (MeCH_2), 55.33 (OMe & C^{e}), 60.43 (C^{c}), 62.03, 61.11, 62.14, 62.31 (C-6 Man, C-6 Man' C-6 Man'' & C^{d}), 64.31, 64.36, 64.41, 64.48 (4 x br, C-6 Gal, C-6 Gal', C-6 Gal'' & CH_2OP), 68.16, 68.87 (C-4 Gal, C-4 Gal' & C-4 Gal''), 69.41 (C-3 Man), 69.48, 69.67 (C-3 Man' & C-3 Man''), 69.90, 69.97, 70.04 (C-5 Man, C-5 Man' & C-5 Man''), 70.39, 70.44, 70.46 (C-2 Gal, C-2 Gal' & C-2 Gal''), 70.59 (br, C-2 Man' & C-2 Man''), 70.92 (C-2 Man), 71.81, 71.88, 71.96 (C-5 Gal, C-5 Gal' & C-5 Gal''), 72.47, 72.58, 72.70 (C-3

Gal, C-3 Gal' and C-3 Gal''), 72.96, 73.20 (C-4 Man' & C-4 Man''), 73.96 (C-4 Man),
93.94, 94.04 (2 x br, C-1 Man' & C-1 Man''), 98.35 (C-1 Man), 101.03 (C-1 Gal'),
101.22, 101.27 (C-1 Gal & C-1 Gal''), 128.24-133.15 (Ph), 165.02-167.78 (PhC=O) and
173.58 (CH₂CONH).

³¹P NMR (CDCl₃): δ_p -4.66, -4.42 (P & P') and -0.59 (P'').

Methyl β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranoside 6-(6-*N*-biotinylaminohexyl phosphate), triethylammonium salt **50**



To a solution of compound **44** (82 mg, 0.055 mmol) in MeOH (20 ml) was added 4.6M NaOMe in MeOH (200 μ l), and the mixture was kept at rt for 20 h. Dowex 50W-X4 (H^+) resin was added to deionise the reaction mixture, then the resin was filtered off, and the filtrate was neutralised with trimethylamine. After concentration, water (5 x 10 ml) was evaporated off from the residue to remove methyl benzoate. This gave the biotinylated disaccharide monophosphate **50** (44.4 mg, 0.052 mmol, 95 %).

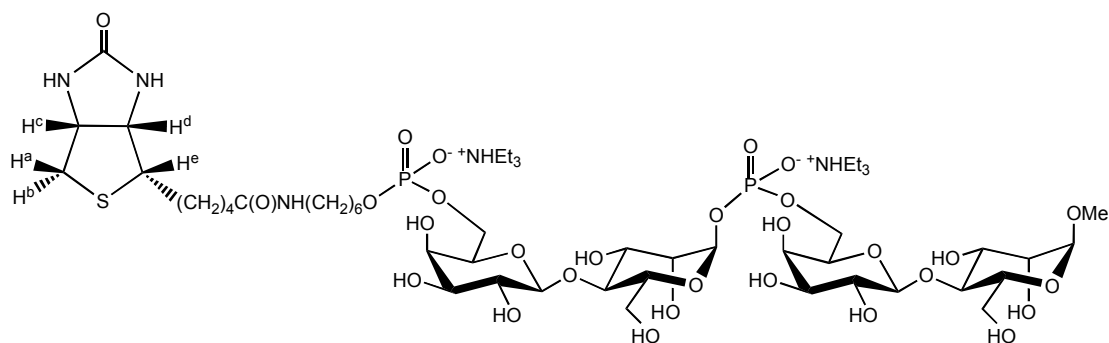
1H NMR ($D_2O + C_5D_5N$): δ_H 0.85-1.65 (21H, m, 3 x $MeCH_2$ & 7 x CH_2), 2.15 (2H, t, J 7.3, CH_2CO), 2.70 (1H, d, $J_{a,b}$ 12.9, H^b), 2.85 (1H, dd, $J_{a,c}$ 4.7, H^a), 3.02 (1H, m, H^e), 3.09-3.16 (8H, m, 3 x $MeCH_2$ & CH_2N), 3.30 (3H, s, OMe), 3.72 (1H, m, H-5), 3.88 (1H, dd, $J_{2',3'}$ 9.9, H-3'), 3.91-4.12 (9H, m, H-2, H-3, H-4, H-6a, H-6b, H-2', H-5' & CH_2OP), 4.18 (1H, dd, $J_{d,e}$ 4.5, H^d), 4.20 (1H, d, $J_{3',4'}$ 3.4, H-4'), 4.30 (2H, m, H-6a' & H-6b'), 4.45 (1H, m, $J_{c,d}$ 8.0, H^c), 4.65 (1H, d, $J_{1',2'}$ 7.9, H-1') and 4.79 (1H, d, $J_{1,2}$ 1.4, H-1).

^{13}C NMR ($\text{D}_2\text{O} + \text{C}_5\text{D}_5\text{N}$): δ_{c} 7.83 (MeCH_2), 24.50, 25.00, 25.67, 27.40, 27.78, 28.17 (6 x CH_2), 29.65 (d, $J_{\text{C,P}}$ 7.8, $\text{CH}_2\text{CH}_2\text{OP}$), 35.11 (CH_2CO), 38.73(CH_2N), 39.45 (C^{ab}), 45.96 (MeCH_2), 54.06 (OMe), 55.03 (C^{e}), 59.56 (C^{c}), 60.09 (C-6), 61.35 (C^{d}), 64.07 (br, C-6'), 65.41 (d, $J_{\text{C,P}}$ 5.5, CH_2OP), 68.06 (C-4'), 69.13 (C-2), 69.38 (C-3), 70.69 (C-2'), 70.84 (C-5), 72.60 (C-3'), 73.94 (d, $J_{\text{C,P}}$ 7.1, C-5'), 77.63 (C-4), 100.24 (C-1), 103.49 (C-1'), 164.31 (NHCONH) and 174.63 (CH_2CONH).

^{31}P NMR ($\text{D}_2\text{O} + \text{C}_5\text{D}_5\text{N}$): δ_{p} -0.32.

High resolution ES-MS(-): found m/z 760.2733 [$\text{M} - \text{Et}_3\text{N} - \text{H}$] $^-$ ($\text{C}_{35}\text{H}_{67}\text{N}_4\text{O}_{16}\text{PS}$ requires M : 862.4010; $\text{C}_{29}\text{H}_{51}\text{N}_3\text{O}_{16}\text{PS}^-$ requires m/z 760.2733).

Methyl β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranoside 6-[β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranosyl phosphate 6-(6-*N*-biotinylaminohexyl phosphate)], bistriethylammonium salt **51**



To a solution of compound **47** (57 mg, 0.022 mmol) in MeOH (15 ml) was added 4.6M NaOMe in MeOH (150 μ l), and the mixture was kept at rt for 20 h. Dowex 50W-X4 (H^+) resin was added to deionise the reaction mixture, then the resin was filtered off, and the filtrate was immediately neutralised with trimethylamine. After concentration, water (5 x 10 ml) was evaporated off from the residue to remove methyl benzoate. This gave the biotinylated tetrasaccharide diphosphate **51** (29.5 mg, 0.0216 mmol, 98 %).

1H NMR ($D_2O + C_5D_5N$): δ_H 1.05-1.83 (14H, m, 7 x CH_2), 1.34 (18H, t, 6 x $MeCH_2$), 2.33 (2H, t, J 7.2, CH_2CO), 2.82 (1H, d, $J_{a,b}$ 12.8, H^b), 2.95 (1H, dd, $J_{a,c}$ 4.7, H^a), 3.12 (1H, m, H^e), 3.17-3.29 (14H, m, 6 x $MeCH_2$ & CH_2N), 3.32 (3H, s, OMe), 3.81 (1H, m, H-5 Man), 4.02-4.09 (3H, m, H-3 Gal, H-5 Man' & H-3 Gal'), 4.11-4.40 (18H, m, H-2, H-3, H-4, H-6a, H-6b Man, H-2, H-4, H-5, Gal, H-3, H-4, H-6a, H-6b Man', H-2, H-4, H-5

Gal', CH₂OP & H^d), 4.45-4.58 (4H, m, H-6a H-6b Gal, H-2 Man', H-6a H-6b Gal' & H^c), 4.82, 4.83 (2H, 2 x d, $J_{1,2}$ 7.8, $J_{1,2}$ 7.5, H-1 Gal & H-1 Gal'), 4.88 (1H, br, H-1 Man) and 6.05 (1H, br d, $J_{1,P}$ 7.9, H-1 Man'),

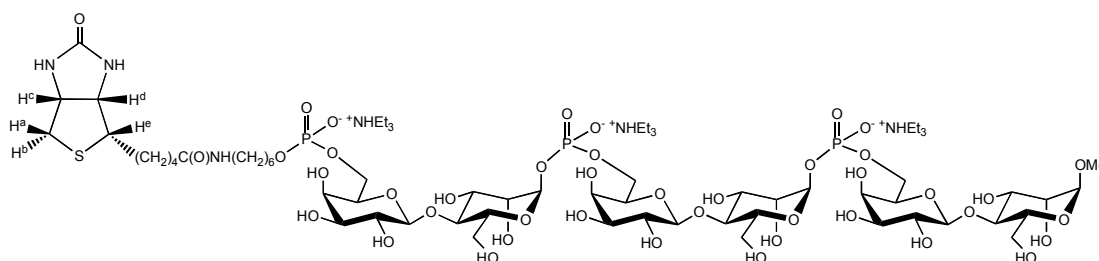
¹H NMR (D₂O) (for bis-ammonium salt of compound **51**): δ_H 1.20-1.65 (14H, m, 7 x CH₂), 2.14 (2H, t, J 7.2, CH₂CO), 2.68 (1H, d, $J_{a,b}$ 13.2, H^b), 2.89 (1H, dd, $J_{a,c}$ 5.0, H^a), 3.08 (2H, m, CH₂N), 3.23 (1H, m, H^e), 3.30 (3H, s, OMe), 3.43, 3.45 (2H, 2 x dd, $J_{2,3}$ 10.3, H-2 Gal & H-2 Gal'), 3.57 (2H, dd, $J_{3,4}$ 3.4, H-3 Gal & H-3 Gal'), 3.63 (1H, m, H-5 Man), 3.88-3.97 (21H, m, H-2, H-3, H-4, H-6a, H-6b Man, H-4, H-5, H-6a, H-6b Gal, H-2, H-3, H-4, H-5, H-6a H-6b Man', H-4, H-5, H-6a, H-6b Gal' & CH₂OP), 4.31 (1H, dd, $J_{d,e}$ 4.5, H^d), 4.34, 4.35 (2H, 2 x d, $J_{1,2}$ 7.9, H-1 Gal & H-1 Gal'), 4.50 (1H, dd, $J_{c,d}$ 7.9, H^c), 4.66 (1H, d, $J_{1,2}$ 1.5, H-1 Man) and 5.33 (1H, dd, $J_{1,P}$ 7.7, $J_{1,2}$ 1.9, H-1 Man').

¹³C NMR (D₂O + C₅D₅N): δ_C 7.80 (MeCH₂), 24.48, 24.95, 25.65, 27.35, 27.73, 28.13 (6 x CH₂), 29.62 (d, $J_{C,P}$ 7.3, CH₂CH₂OP), 35.07 (CH₂CO), 38.71 (CH₂N) 39.43 (C^{ab}), 45.90 (MeCH₂), 54.01 (OMe), 55.00 (C^e), 59.51 (C^c), 60.08, 60.12 (C-6 Man & C-6 Man'), 61.30 (C^d), 63.77 (d, $J_{C,P}$ 4.0, C-6 Gal), 64.27 (d, $J_{C,P}$ 5.3, C-6 Gal'), 65.37 (d, $J_{C,P}$ 4.0, CH₂OP), 67.95, 68.00 (C-4 Gal & C-4 Gal'), 68.71 (C-3 Man'), 69.04 (C-2 Man), 69.31 (C-3 Man), 69.78 (d, $J_{C,P}$ 8.6, C-2 Man'), 70.64, 70.67 (C-2 Gal & C-2 Gal'), 70.79 (C-5 Man), 72.16 (C-5 Man'), 72.46, 72.56 (C-3 Gal & C-3 Gal'), 73.80 (d, $J_{C,P}$ 7.9, C-5 Gal & C-5 Gal'), 77.26 (C-4 Man'), 77.52 (C-4 Man), 95.65 (d, $J_{C,P}$ 6.6 C-1 Man'), 100.17 (C-1 Man), 103.38, 103.47 (C-1 Gal & C-1 Gal'), 164.32 (NHCONH) and 174.60 (CH₂CONH).

³¹P NMR (D₂O + C₅D₅N): δ_P -2.33 (P) and 0.40 (P').

High resolution ES-MS(-): found m/z 1164.3488 $[M - 2Et_3N - H]^-$ ($C_{53}H_{103}N_5O_{29}P_2S$ requires M : 1367.5935; $C_{41}H_{72}N_3O_{29}P_2S^-$ requires m/z 1164.3453).

Methyl β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranoside 6-{ β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranosyl phosphate 6-[β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-mannopyranosyl phosphate 6-(6-*N*-biotinylaminohexyl phosphate)]}, tris(ethylammonium) salt **52**



To a solution of compound **49** (35 mg, 0.009 mmol) in MeOH (10 ml) was added 4.6M NaOMe in MeOH (100 μ l), and the mixture was kept at rt for 20 h. Dowex 50W-X4 (H^+) resin was added to deionise the reaction mixture, then the resin was filtered off, and the filtrate was immediately neutralised with trimethylamine. After concentration, water (5 x 10 ml) was evaporated off from the residue to remove methyl benzoate. This gave the biotinylated hexasaccharide triphosphate **52** (15.2 mg, 0.0081 mmol, 90 %).

1H NMR (D_2O): δ_H 0.80-1.49 (14H, m, 7 x CH_2), 1.50 (27H, t, 9 x $MeCH_2$), 2.05 (2H, m, CH_2CO), 2.29 (2H, m, H^a & H^b), 2.86-2.99 (21H, m, 9 x $MeCH_2$, CH_2N & H^e), 3.20 (3H, s,

OMe), 3.40-4.09 (40H, m, H-2, H-3, H-4, H-5, H-6a, H-6b Man, H-2, H-3, H-4, H-5, H-6a, H-6b Gal, H-2, H-3, H-4, H-5, H-6a, H-6b Man', H-2, H-3, H-4, H-5, H-6a, H-6b Gal', H-2, H-3, H-4, H-5, H-6a, H-6b Man'', H-2, H-3, H-4, H-5, H-6a, H-6b Gal'', CH₂OP, H^c & H^d), 4.39, 4.40, 4.41 (3H, 3 x d, $J_{1,2}$ 7.5, H-1 Gal, H-1 Gal' & H-1 Gal''), 4.59 (1H, d, $J_{1,2}$ 1.5, H-1 Man), 5.12 (1H, m, H-1 Man' or Man'') and 5.48 (1H, m, H-1 Man' or Man'').

¹³C NMR (D₂O) (selected signals): δ_c 8.04 (MeCH₂), 39.62 (C^{ab}), 46.36 (MeCH₂), 54.50 (OMe), 59.83, 60.28 (C-6 Man, C-6 Man' C-6 Man'' & C^d), 62.43 (br, C-6 Gal, C-6 Gal' & C-6 Gal''), 64.11 (br, CH₂OP), 94.84 (br, C-1 Man' & C-1 Man''), 94.41 (C-1 Man) and 103.29, 104.23 (C-1 Gal, C-1 Gal' & C-1 Gal'').

³¹P NMR (D₂O): δ_p -2.02 (P), 0.50 (P') and 0.73 (P'').

High resolution ES-MS(-): found m/z 1584.4125 [M - 3Et₃N - H + NH₃]⁻

(C₇₁H₁₃₉N₆O₄₂P₃S requires M: 1872.7859; C₅₃H₉₆N₄O₄₂P₃S⁻ requires m/z 1585.4438),

1606.3834 [M - 3Et₃N - 2H + K]⁻ (C₅₃H₉₂KN₃O₄₂P₃S⁻ requires m/z 1606.3731),

1628.3928 [M - 3Et₃N - 3H + Na + K]⁻ (C₅₃H₉₁KN₃NaO₄₂P₃S⁻ requires m/z 1628.3551),

1633.9478 [M - 3Et₃N - 4H + 3Na]⁻ (C₅₃H₉₀N₃Na₃O₄₂P₃S⁻ requires m/z 1634.3631) and

1669.9165 [M - 2Et₃N - H]⁻ (C₅₉H₁₀₈N₄O₄₂P₃S⁻ requires m/z 1669.5377).

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